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Lysogeny: Practical Applications and New Discoveries.

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Lysogeny: Practical Applications and New Discoveries.

by

Lauren McDaniel

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
College of Marine Science
University of South Florida

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environmental mutagenesis, MPIA, prophage induction

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Dedication

This work is dedicated to my husband Tom for making me understand I could really accomplish this task, to my family (McDaniel's, Roeser's, and Lamb's) for helping us all through the long haul, and to my professor John Paul for giving me a chance.

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Lysogeny: Practical Applications and New Discoveries

Lauren McDaniel

ABSTRACT

Part 1:

Prophage induction has been demonstrated to be a sensitive indicator for a wide variety of toxic and mutagenic compounds and, as a consequence, has been utilized for biologically based carcinogen screenings. Fourteen marine bacterial isolates were screened for development into the Marine Prophage Induction Assay (MPIA), for marine samples. The selected isolate (P99-4S3) was identified by 16S rDNA sequencing as *Pseudomonas aeruginosa*. This isolate demonstrated a log-linear response to increasing dose of mutagens, and sensitivity to known environmental contaminants. Field-testing of the assay over two years demonstrated the MPIA would be a useful screening tool for environmental contamination.

Part 2:

The observed resistance of natural populations of *Synechococcus* to viral infection may be due to lysogeny with associated homoimmunity. A thirteen-month study of lysogeny in natural populations of *Synechococcus* demonstrated that lysogeny does occur and exhibits a seasonal pattern.

Experiments were performed along a transect of the Mississippi River plume, which provided a variety of ambient nutrient regimes for comparison of lysogeny in *Synechococcus*. Nutrient amendments did not enable induction and often led to a decrease in viral production. Lysogeny in *Synechococcus* was primarily correlated with ambient host and cyanophage abundance.

Cross-infectivity studies demonstrated cyanophage isolates possess variable virulence. The 35 isolates were examined by transmission electron microscopy (TEM), with 33 identified as myoviruses and two as podoviruses. This dominance of myovirus lytic cyanophage is consistent with prior observations.

Twenty-five *Synechococcus* isolates were screened for prophage induction utilizing the inducing agent Mitomycin C. Eleven isolates demonstrated a statistically significant increase in virus-like particles (VLP's) in treatment samples. No correlation was observed between their resistance to lytic viral infection and prophage induction. Isolate P99-14, with consistently high levels of prophage induction, was investigated further. In contrast to lytic cyanophage, the induced cyanophage is non-tailed. Differential staining and nuclease digestion experiments indicate that the induced particle contains single-stranded DNA.

Environmental conditions potentially leading to prophage induction were investigated with *Synechococcus* cultures and natural populations. The isolate P99-14 demonstrated that high, continuous light caused prophage induction. Natural populations determined that shifts in salinity, temperature and phosphate are not triggers of prophage induction.

"It is astonishing but true-microbes are responsible for maintaining life on Earth. By driving global cycles of oxygen, carbon, and other essential elements, microbes support the remarkable diversity of animals and plants that have played an essential role in the creation of the fertile land that sustains crops and livestock and, therefore, sustains human populations. Even within our own bodies, bacteria are necessary for digestion and absorption of nutrients and for educating our immune systems. Life on earth has thrived because of the activities of microorganisms, and in their absence that life would cease to exist." (Buckley, 2002)

INTRODUCTION

Microbes, particularly those in the sea, are essential to the functioning of Earth ecosystems. The total number of prokaryotes worldwide is estimated to be around $4-6 \times 10^{30}$ cells with a total carbon content of 350-550 Pg (1 Pg = 10^{15} g), about 60-100% of the total carbon content of terrestrial plants (Whitman, Coleman, and Wiebe, 1998). Although bacteria are not visible to the naked eye the ocean contains more than 3×10^{28} , making them the major source of biomass (Copley, 2002). Bacteria are also known to drive planetary scale biogeochemical cycles including those of carbon and nitrogen. In many environments microbes are the only source of essential nutrients for higher organisms (Madigan, Martinko, and Parker, 2000).

Despite the obvious importance of microbes, relatively little is known about the role of the majority of bacteria in the sea. Only a small fraction of the total number of microorganisms in the ocean have been cultured and characterized (about 0.1%-1%) (Colwell and Grimes, 2000).

In marine environments, heterotrophic bacteria are an important component of plankton. These microorganisms directly utilize the dissolved products of primary production, termed dissolved organic matter (DOM), and recycle energy and limiting nutrients in a process known as the microbial loop (Azam et al., 1983). This cycle forms an essential component of marine food webs.

Autotrophic picocyanobacteria are another important group of prokaryotic microbes in the marine environment. These organisms have an ancient lineage and are believed to be the first to develop oxygenic photosynthesis. These life forms or their close relatives were responsible for oxygenating the Earth's atmosphere over 3.5 billion years ago, causing an oxidized global ocean by 2.1 billion years ago (Rye and Holland, 1998). The unicellular forms of cyanobacteria numerically dominate the oligotrophic areas of the ocean, yet were only first observed in 1979 (Waterbury et al., 1979). These simple plant-like microbes continue to be vital for the Earth

ecosystem today. More than one-half of global primary productivity has been estimated to occur in the oceans, the majority attributable to unicellular forms of cyanobacteria (Chisholm, 2000; Whitman, Coleman, and Wiebe, 1998).

There is a continually emerging appreciation of the unexplored phylogenetic and metabolic diversity in marine microbial communities. Culture-independent analyses of marine samples are a significant new method of exploring this diversity of marine microbes. An entire new group of ubiquitous α -proteobacteria called SAR11 was identified and later cultivated based on ribosomal RNA gene (rRNA) gene cloning and sequencing (Rappé et al., 2002). This same technique has been used to explore the diversity and abundance of the poorly understood eukaryotic picoplankton group (Guillou et al., 1999).

The marine Archaea are another unusual group of microbes that was uncovered by culture-independent techniques. Marine Archaea have been discovered in wide-ranging environments throughout the global ocean. Molecular techniques such as fluorescence in-situ hybridization (FISH) have documented blooms of marine archaea in North Sea surface waters, with archaeal types sometimes exceeding 30% of the total picoplankton abundance (Pernthaler et al., 2002).

The genetic approach can also be used in conjunction with functional genes, such as a gene for ammonia oxidation (*amoA*) or carbon fixation (*rbcL*), to determine the distribution of specific metabolic activities of interest (Pichard, Frischer, and Paul, 1993; Ward and O'Mullan, 2002). This tactic allows determination of the distribution as well as the diversity and phylogeny of a functional group of microbes.

Another culture-independent technique for studying marine microbes is termed metagenomics. This technique involves the direct isolation of genomic DNA from a natural environment of interest. Community metagenomic analysis includes the sequencing, assembly and analysis of the entire microbial community. This approach has been used to examine the microbial communities from environments as divergent as the Sargasso Sea (Ventner et al., 2004) to an acid mine drainage pool (Tyson et al., 2004). Totally novel metabolic processes, such as a unique type of photic energy capture based on bacterial rhodopsin, have only recently

been revealed by these methods (Béjà et al., 2000; Kolber et al., 2000). This finding was revealed from uncultivated microbes, based on a genomic fragment, and was later confirmed by spectral analysis of cell membrane proteins (Béjà et al., 2001). These organisms are not only unusual, but they have been confirmed to be phylogenetically diverse and may account for up to 11% of the marine microbial community (Béjà et al., 2002; Karl, 2002). Culture-independent analyses of bacterial diversity have indicated that the number of bacterial types is about triple the diversity estimated based on culture-dependent studies (Stackebrandt and Embley, 2000).

These new methodologies appear to be replacing the more conventional culture-based microbiological methods due to advances in molecular methods and the inherent difficulty in cultivating marine microbes. Future innovations in metagenomic analysis will need to include improvements in method development, including more diverse hosts to allow investigation of heterologous gene expression and better tools for bioinformatic analysis [reviewed in (Handelsman, 2004)].

Just as microbes control many ecosystem processes, viruses exert a major control over bacteria. Viruses have been found to be the most abundant form of life on the planet. Marine viruses have also been verified to be active components of both the water column and the sediments (Hewson et al., 2001; Middelboe and Glud, 2003; Wommack and Colwell, 2000). Large numbers of viruses have also been observed in marine samples from depths as great as 5000m (Hara et al., 1996). Although viruses are abundant in both the water column and the sediments, it appears that the factors controlling their distribution differ between these two environments (Hewson et al., 2001; Paul et al., 1993).

Of the viruses studied so far, the majority infect bacteria. Therefore, bacteriophages (also called phages) are the largest known viral group (Ackermann and DuBow, 1987). Determining how bacteria and viruses function together within the microbial loop has taken on new importance in the current context of global warming. Understanding how microbes influence climate by their cycling of essential nutrients may help us predict, or perhaps even counter, some of the effects of large-scale anthropogenic carbon and nitrogen releases into the environment (Copley, 2002; Wommack and Colwell, 2000).

Bacteriophages have two differing modes of existence: lytic infection and lysogeny. During lytic infection a virus injects its nucleic acid into a host cell, replicates utilizing the host's metabolic machinery, and then lyses the host to release progeny viruses. In lysogeny the virus injects its nucleic acid but does not begin replication. The virus becomes integrated into one of the host replicons (chromosome or another integrated phage genome) and is thereafter replicated along with the host, during cell division (Ackermann and DuBow, 1987). Once the virus becomes integrated into the host it is then called a prophage. Viruses with the ability to integrate with their host in this way are called temperate phages, as opposed to intemperate, or lytic bacteriophages. Although temperate phages generally integrate into the host chromosome, they are occasionally maintained as a plasmid.

Lysogens are usually detected experimentally by exposing the host to a mutagen or metabolic inhibitor. This treatment will lead to excision of the prophage from the host. Although prophages have historically been detected experimentally, several recent discoveries of prophage have resulted from bacterial genome analysis.

This dissertation explores two facets of the process of lysogeny in marine environments. The first aspect of this study investigates practical applications of lysogeny based on sensitivity of the phage-host system to DNA damage and metabolic insults. The second aspect explores potential ecological implications of lysogeny in autotrophic marine *Synechococcus*.

CHAPTER 1

LITERATURE REVIEW

MICROBIAL LOOP

Brief History and Description of the Microbial Loop

For most of the history of marine science, bacteria and viruses were thought to have a relatively small impact on processes in marine environments. Microbes were considered to function primarily as decomposers. One early clue to the recognition of the importance of bacteria in aquatic systems was the demonstration of high metabolic activity in the bacterial size fraction of pelagic marine samples (Azam, 1977).

Based on Azam's (1977) observations, the microbial loop theory was developed (Azam et al., 1983). This theory contended that bacteria are not simply decomposers and remineralizers of waste products, but primary consumers themselves, directly utilizing an estimated 10-50% of the carbon fixed by photosynthesis. The theory contends that organisms tend to consume particles about one order of magnitude smaller than themselves. Because of their small size, bacteria are ideally suited to utilize the dissolved organic matter (DOM) produced by phytoplankton. This energy is then "rather inefficiently returned to the main food chain via a microbial loop of bacteria-flagellates-microzooplankton" (Azam et al., 1983).

Shortly afterward, improved experimental techniques allowed the observation of high concentrations of bacteria and viruses in seawater (Bergh et al., 1989). In addition, improved methods for measuring the rate of secondary production provided corroboration of the high metabolic activity of marine bacteria (Chin-Leo and Kirchman, 1988). The confirmation of high abundances and activity of bacteria led to general acceptance of the microbial loop theory. This paradigm shift has led to the understanding of the significance of microbes in the marine

environment for the recycling of energy within the DOM pool and that aquatic environments are more a “food web” than a chain (Fig 1-1) (Meyers, 2000).

Impacts of the Microbial Loop

Initial estimates indicated that primary production and bacterial production are usually tightly coupled and that somewhere between 20-50% of primary productivity is consumed by bacteria within the microbial loop (Azam, Smith, and Hagström, 1994). However, the coupling between these two processes is variable and may determine overall ecosystem productivity. The presence of a dominant microbial loop has been associated with diminished fish production, while a highly productive fishery was ascribed to an uncoupling of primary and secondary production (Azam, 1998).

Continuing investigation of the microbial fraction of seawater has revealed the primary role of ciliates and heterotrophic flagellates as grazers, providing the intermediate predator level within the microbial loop. Modeling experiments have suggested the importance of zooplankton in controlling fluxes of nutrients within and out of the microbial loop, both to higher trophic levels and in export from the euphotic zone (Steele, 1998). More recent estimates of secondary production based on modeling experiments calibrated with known productivity estimates suggest that only 10-15% of primary productivity is consumed by bacterial production in oligotrophic areas (Anderson and Ducklow, 2001). This work confirmed the previously observed close coupling between primary and secondary production as well as supporting the importance of zooplankton grazing to microbial loop processes. In oligotrophic areas zooplankton grazing may provide a significant supply of DOM for bacterial consumption. The contribution of zooplankton grazing to DOM was supported by evidence of lower than expected release of DOM by phytoplankton, which is commonly thought to be a major food source for bacteria.

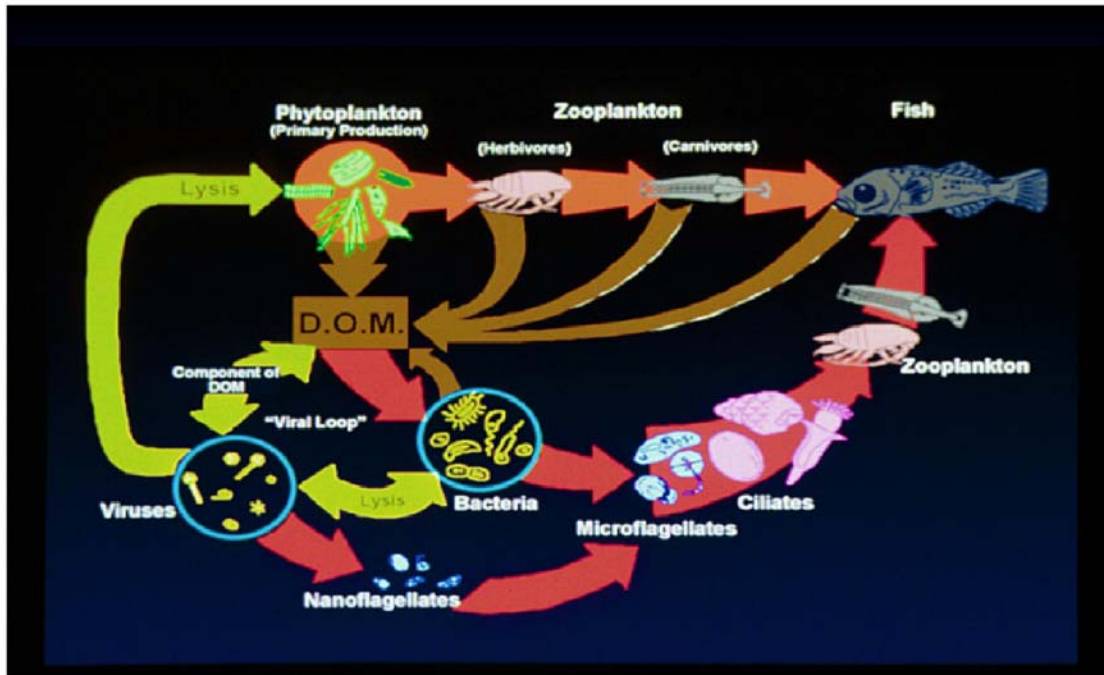


Figure 1-1: Illustration of the Microbial Loop.

Subsequent to the development of the initial microbial loop theory, research has focused on the importance of processes such as the formation of colloids and marine snow as “hot spots” for microbial activity in the water column (Chin, Orellana, and Verdugo, 1998). These micro aggregates may support the bulk of bacterial production in seawater as well as providing microenvironments allowing anaerobic processes in the oxygenated water column (Meyers, 2000).

SYNECHOCOCCUS

Classification

Cyanobacteria are ubiquitous photosynthetic prokaryotic organisms found in marine, terrestrial and sedimentary environments throughout the Earth. Cyanobacteria have an extremely long evolutionary history, which has allowed them to adapt to many types of habitat,

including extreme environments such as thermal springs (Whitton and Potts, 2000).

Cyanobacteria have a wide morphological diversity, ranging from simple unicellular forms dividing by binary fission to complex filamentous and branching forms with specialized cell types and complex life cycles. The dominant nutritional mode in cyanobacteria is oxygenic photosynthesis, similar to land plants (Waterbury et al., 1986b). This dissertation focused on marine *Synechococcus*, which is one of the simplest, yet ecologically important cyanobacterial types.

Synechococcus is the general form-genus group name applied to all unicellular cyanobacterial forms. Marine *Synechococcus* are unicellular, generally coccoid in shape and range in size from 0.6-1.2 μ m in size. They demonstrate the prokaryotic features of a gram-negative type of cell wall consisting of a peptidoglycan layer and an outer membrane. Similarly to all cyanobacteria, they contain a chlorophyll a photosynthetic reaction center. In addition, *Synechococcus* strains contain antenna pigments composed of phycobiliproteins, which is a unique adaptation for preferential absorption of blue light by water. The phycobiliprotein pigments come in two general types, which vary in absorbance spectrum, phycoerythrin and phycocyanin.

Marine forms of *Synechococcus* fall into two distinct phenotypic subgroups. The first group contains both phycobiliprotein antenna pigments and has a requirement for elevated salt levels for growth and cannot tolerate fresh water. The other physiological type lacks phycoerythrin and can tolerate but does not require elevated salt for growth. The type of antenna pigment and the phycoerythrin to phycocyanin ratio has been used in the past as part of a general classification scheme for *Synechococcus* strains, but has fallen out of favor as the pigment ratio has not proven to be a monophyletic trait.

The *Synechococcus* types that carry only phycocyanin antenna pigments (Bergey's clusters 1,2 and 3) appear green in color. Some marine strains may contain both phycocyanin and phycoerythrin (clusters 4 and 5), therefore may range in color from an almost olive green to quite red depending on the ratio of the phycoerythrin to phycocyanin. Some *Synechococcus* types have a fixed pigment ratio and some isolates have been observed to change their pigment ratios depending on ambient light conditions. Natural populations of *Synechococcus* cells in natural populations can also photo acclimate by increasing their abundance of pigments with

depth (Olson et al., 1990). *Synechococcus* is readily identifiable in natural samples since cells containing phycoerythrin auto-fluoresce a brilliant yellow-orange when observed with an epifluorescence microscope under blue excitation.

Synechococcus cells also have a characteristic ultrastructural feature. Under transmission electron microscopy (TEM), they contain stacked photosynthetic structures called thylakoids (Fig. 1-2). They are located in the periphery of the cell, just inside the cytoplasmic membrane (Herdman et al., 2001). In *Synechococcus* these structures are separated by about 40-50 nm due to the attached phycobilisomes, which is an interesting pyramid shaped structure consisting of the antenna pigments and the chlorophyll-a photosynthetic reaction center. The very closely related unicellular marine cyanobacterium *Prochlorococcus* does not contain these structures. Ting and Roco (2002) hypothesized, based on phylogenetic analyses, that the photosynthetic apparatus of *Prochlorococcus* evolved without phycobilisomes in response to iron limitation.

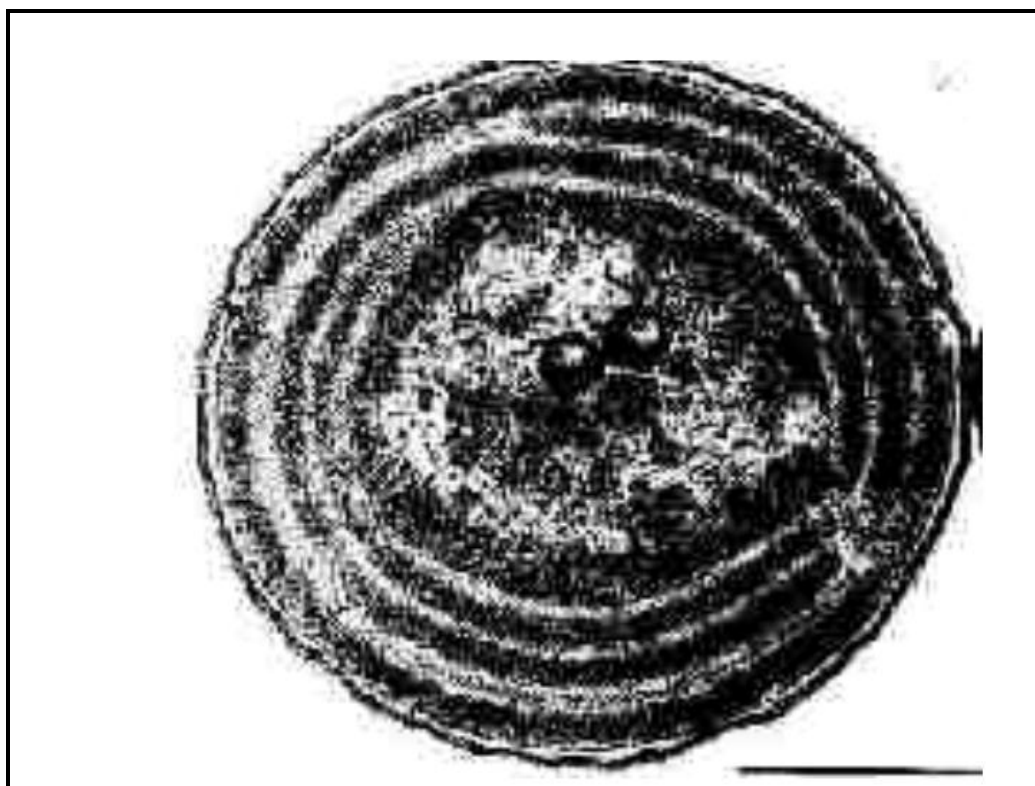


Figure 1-2: Cross-section of a *Synechococcus* cell with concentric thylakoids clearly visible. Also note the carboxysomes in the center of the cell.

Initially, classification into the provisional Genus *Synechococcus* was based on the phenotypic characters of unicellular morphology under 3µm, division by binary fission in one plane and peripheral, concentric thylakoids. The genus was further subdivided into five subgroups or clusters based on salt requirement, pigments and genome G+C content (Holt et al., 1994). However, after this classification was developed, analysis of restriction fragment length polymorphisms (RFLP's) of several genes determined that G+C content was not a reliable criteria for classification (Douglas and Carr, 1988).

Although *Synechococcus* is treated as a single genus based on phenotype, substantial genetic diversity has been observed within this group. Phylogenetic analysis will most likely permit further subdivision into different genera (Douglas and Carr, 1988; Holt et al., 1994). In addition to the genetic diversity, morphologically similar forms of *Synechococcus* have been observed to have variable physiology, including variable salt tolerance, variable pigment content, differing cell-cycle behavior (Binder and Chisholm, 1995) and the presence of swimming motility (Waterbury et al., 1985; Willey and Waterbury, 1989).

Several recent studies have contributed to the determination of the genetic and phenotypic characters providing a more meaningful classification scheme for marine *Synechococcus*. The commonly used method of prokaryotic classification based on 16S rRNA genes revealed that all cyanobacteria fall within eight major lineages. *Synechococcus* strains affiliated with five of these deeply branching cyanobacterial lineages indicating that the unicellular morphology is a polyphyletic phenotypic character (Honda, Yokota, and Sugiyama, 1999). Robertson (2001) postulated that the unicellular morphology of *Synechococcus* is most likely an ancient morphotype from which more complex types of cyanobacteria evolved. (Robertson, Tezuka, and Watanabe, 2001) Interestingly, all of the marine *Synechococcus* isolates fell within a single clade. However, the 16S phylogeny does not provide adequate resolution to differentiate between *Synechococcus* and *Prochlorococcus*. A number of other genes have been analyzed to provide a higher level of genetic resolution within these groups.

One of the most widely used genes for phylogeny, after 16S rDNA, is the gene encoding the carbon fixation enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase, also known as

RuBisCO. The gene for the large subunit of this enzyme (*rbcL*) is very useful for phylogenetic analysis of autotrophic microorganisms including cyanobacteria because it provides higher resolution than the 16S rDNA gene. The phylogeny based on this gene is consistent with the 16S rDNA phylogeny and is identical to the groupings based on carboxysome phylogeny (Badger, Hanson, and Price, 2002; Cannon et al., 2002; Honda, Yokota, and Sugiyama, 1999). Additionally, the α type carboxysome corresponds with form 1A Rubisco and the β type of carboxysome corresponds with the form 1B Rubisco. Thus, these phenotypic characters might be useful for incorporation into the current classification scheme for *Synechococcus*. *RbcL* gene expression has already been used effectively for studying the composition and activity of natural populations of both marine and phytoplankton communities (Paul, Alfreider, and Wawrik, 2000; Paul et al., 1999; Pichard, Frischer, and Paul, 1993; Wyman, 1999; Xu and Tabita, 1996).

Several studies have utilized the gene sequence for the DNA-dependent RNA polymerase (*rpoC1*) to compare marine unicellular cyanobacteria. This gene provides excellent resolution between *Synechococcus* and *Prochlorococcus* (Palenik, 1994). It was also useful in examination of *Synechococcus* groups with differing phenotypic traits to determine if pigment ratios, a phenotypic character used in classification, are a genetically meaningful way of grouping isolates (Toledo and Palenik, 1997; Toledo, Palenik, and Brahamsha, 1999). The most striking finding of this research has been that the pigment ratio is not genetically informative and that isolates capable of swimming motility form a monophyletic group based on *rpoC1*.

Another comparison between *Synechococcus* and *Prochlorococcus* was performed utilizing photosystem antenna protein (*psbB*) and cytochrome complex protein (*petB* and *D*) genes (Urbach et al., 1998). These genes were selected because they are single copy genes unique to photosynthetic organisms, yet could provide greater phylogenetic resolution than the 16S gene. This analysis resulted in the similar conclusions that unicellular morphotype and pigment ratios are unreliable taxonomic indicators and also demonstrated that *Synechococcus* and *Prochlorococcus* have diversified relatively recently.

Interestingly, the molecular ecology of *Prochlorococcus* is fairly straightforward. The molecular phylogeny separates *Prochlorococcus* into two distinct clades corresponding to well-

defined high light and low light ecotypes (Scanlan and West, 2002). However, *Synechococcus* appears to be decidedly more complex and diverse in both physiology and habitat. Some *Synechococcus* types such as marine clusters 3 and 5.2 (old classification B and C), appear to be sparsely sampled and under represented in current phylogenies (Scanlan and West, 2002).

A study of high light inducible genes (*hli*) in several cyanobacterial types supported the monophyletic nature of the marine *Synechococcus* group (Bhaya et al., 2002). However, this analysis differed from the 16S phylogeny in some of the freshwater species of cyanobacteria. The authors suggested that these differences could be useful in determining which environmental pressures, such as light intensity, drive genetic adaptation.

The current classification scheme of Bergey's Systematic Bacteriology (Herdman et al., 2001) incorporates most of the previously described phenotypic characters and genetic data. Although the classification scheme is still considered provisional, it has been well supported by phylogenetic studies of different genetic loci (Herdman et al., 2001). The main characters currently used for classification into the *Synechococcus* group include: prokaryotic organism, oxygenic photosynthetic metabolism, group placement by 16S rDNA phylogeny, spherical to rod-shaped morphology, 0.6-2.1 μm in diameter, presence of peripheral thylakoids and division by binary fission.

Characterized strains from wide-ranging environments are further subdivided into five "clusters" with sub clusters totaling nine distinct groups based on salt requirement, cell size, and DNA-DNA hybridization studies. The isolates utilized in this dissertation fall into cluster 5.1, which is equivalent to marine cluster A under the previous classification scheme. Isolates in this cluster are obligate marine forms, ranging from 0.6-1.7 μm in diameter and containing phycoerythrin as their main light harvesting (antenna) pigment. In addition, cluster 5.1 contains the *Synechococcus* isolates that have demonstrated swimming motility, although not all isolates in this cluster are motile.

Ecological Importance of *Synechococcus*

A strikingly consistent pattern of phytoplankton diversity has been observed throughout the world's oceans (Irigoien, Huisman, and Harris, 2004). Similar to terrestrial ecosystems, the highest diversity of species was observed in areas of intermediate phytoplankton biomass, while minimum diversity occurred during bloom conditions.

Between these two diversity extremes is that of the oligotrophic ocean, which constitutes the largest portion of the photic zone ecosystem. In this area, two unicellular types of cyanobacteria, *Prochlorococcus* and its close cousin *Synechococcus*, are the dominant phytoplankton species. Due to the vast area of the oligotrophic ocean, both of these organisms are major contributors to oceanic and global primary productivity, with *Synechococcus* alone contributing an estimated 25% (Chisholm et al., 1988; Liu, Nolla, and Campbell, 1997). It has been suggested that the two main reasons why these unicellular types are so dominant within open ocean provinces is both the ability of the unicellular form to withstand the high shear conditions from wind and wave action (Paerl, 2000) and their high surface to volume ratio, allowing rapid nutrient uptake (Button, 2000).

In addition to their ability to fix carbon, cyanobacteria play a vital ecological function by their ability to fix nitrogen. This ability has long been attributed to the filamentous form *Trichodesmium*. Nitrogen fixation is accomplished in the oxygenated water column by both temporal and spatial separation of anaerobic nitrogen fixation and oxygenic photosynthesis (Berman-Frank et al., 2001). However, it has also been demonstrated that some unicellular cyanobacterial forms can perform this function (Falcón et al., 2002; Zehr et al., 2001). Nitrogenase (*nifH*) genes have been identified from unicellular forms of cyanobacteria in both the Atlantic and Pacific oceans (Zehr, Mellon, and Zani, 1998).

Effects of Nutrient Limitation on *Synechococcus*

The Redfield ratio, a classic concept in marine biology, was formulated in 1958 (Redfield, 1958). Redfield (1958) observed that plankton have a set elemental ratio with the proportion of nitrogen to phosphorus (N:P ratio) being approximately 16 based on the number of number of atoms. This ratio was found to be remarkably consistent throughout all ocean environments.

Klausmeier et al (2004) concluded that this fixed stoichiometry is an overall average and can vary by both phytoplankton species and growth conditions. They also found that phytoplankton growing under competitive equilibrium utilize cellular functions that favor a higher cellular N:P ratio. Exponential growth conditions require higher inputs of phosphorus, hence a lower cellular N:P ratio. Therefore, cells in stationary phase of growth would be more severely affected by nitrogen limitation, while those in log phase would be more strongly influenced by phosphate limitation. (Klausmeier et al., 2004)

A great deal of early investigation was performed on the effects of nitrogen (N) limitation in *Synechococcus*. Different isolates of *Synechococcus* have been reported to utilize a wide variety of nitrogen sources including NH_4^+ , NO_3^- , NO_2^- , urea, and amino acids as their sole nitrogen source, as well as having systems for active uptake of these compounds. Under nitrogen deprivation, *Synechococcus* can also degrade its internal store of phycoerythrin pigment as an internal N source (Moore et al., 2002). Therefore it appears that *Synechococcus* is well adapted to tolerate the episodic nutrient pulses and periodic nitrogen limitation, which is characteristic of marine habitats, without adverse effect. In addition, the completed genome sequence of the *Synechococcus* strain WH8102 indicates that at least some strains are capable of utilizing alternative organic sources of nitrogen and phosphorus (Palenik et al., 2003b).

More recently, Lindell and Post (2001) investigated the nitrogen status of natural *Synechococcus* populations by analyzing the level of expression of the gene (*ntcA*) for the nitrogen regulatory protein NtcA. (Lindell and Post, 2001) This study revealed that *Synechococcus* populations were not nitrogen stressed even at environmental nitrogen

concentrations of less than $1\mu\text{M}$. The researchers concluded that their nitrogen requirements of *Synechococcus* are easily met by locally regenerated ammonium.

This conclusion was supported by a recent study of phytoplankton populations in the Gulf of Mexico within an area influenced by nutrient inputs from the Mississippi River plume (Wawrik et al., 2004). This high level of ammonium regeneration was found to support large *Synechococcus* populations as well as high levels of carbon fixation.

Several studies investigated the effects of nutrient enrichment on natural mixed bacterioplankton populations. One of these studies indicated that nutrient stimulation leads to changes in metabolic activity and community composition that differed based on the type of nutrient addition (Tuomi et al., 1995). Amino acid stimulation led to increased viral production with an increased virus to bacteria ratio (VBR). The subsequent addition of phosphate led to an overall increase in bacterial biomass without a proportional increase in viruses. An overall decrease in diversity, based on the number of cell types, was observed with both types of nutrient stimulation. However, a transient drop in the bacterial abundance in the phosphate-amended samples in conjunction with the appearance of "cell ghosts," led to the hypothesis that the phosphate addition may have stimulated prophage induction in a subset of the population, leading to the decreased diversity (Tuomi et al., 1995).

A study of natural bacterial communities in the Gulf of Mexico came to a similar conclusion (Williamson and Paul, 2004). Nutrients were added to seawater samples to determine if prophage induction would result. The authors concluded that nutrient stimulation led to increased lytic viral production rather than prophage induction.

In *Synechococcus*, a high affinity for phosphate, as well as a high maximum uptake rate, have been experimentally observed (Ikeya et al., 1997). These findings led to the hypothesis that phosphate kinetics might explain the dominance of this species in both phosphate-depleted oligotrophic areas as well as areas with episodic inputs of phosphorus (Moutin et al., 2002).

Wilson and Mann (1997) hypothesized that the phosphate status of *Synechococcus* would have profound effects on the outcome of viral infection modulating carbon cycling through the microbial loop. This is not an unreasonable hypothesis since phage have an observed

tendency to utilize components of nutrient transport systems as receptors (Wilson and Mann, 1997). Experimentation with *Synechococcus* cultures under phosphate-deplete and replete growth conditions demonstrated no differences in phage adsorption kinetics, but did indicate marked decreases in burst size when phosphate was limited (Wilson, Carr, and Mann, 1996). The researchers concluded that the cyanophages established lysogeny in response to phosphate limitation of the host.

Further investigation of the conclusions reached by Wilson et al (1996) was performed using natural populations of *Synechococcus* in seawater mesocosms. Following phosphate amendment, a large increase in viral abundance was observed, followed by a collapse in the *Synechococcus* bloom (Wilson, Turner, and Mann, 1998). The researchers suggested that the phosphate addition caused induction of temperate viruses and that nutrient availability may be responsible for the switch from lysogenic to lytic production. However, the nutrients could also have stimulated an increase in host growth followed by a lytic infection cycle. Despite these contradictory possible conclusions, the data suggested some link between supply of phosphate and viral infection kinetics.

VIRUSES IN THE MARINE ENVIRONMENT

High Abundance of Viruses

Bacterial viruses were actually discovered twice at the beginning of the century, in 1915 by F.W. Twort, and in 1917 by Félix d'Hérelle (Ackermann, 1997). However, the discovery is generally credited to Félix d'Hérelle because he was the first one to recognize the infectious nature of bacteriophages and postulate their intracellular replication. In 1946 Zobell first isolated bacteriophages from seawater using a plaque assay method based on an enteric host (Zobell, 1946). By 1955 Spencer provided the first detailed description of a bacteriophage isolated from the sea (Spencer, 1955).

The abundance of viruses in the marine environment was believed to be very low based on early studies using plaque assays. Viruses were considered to be of no consequence to the ecology of marine waters for many years. Marine samples were then observed using the transmission electron microscope (TEM), radically altering this view (Bergh et al., 1989). Viruses were found in abundances up to $2.5 \times 10^8 \text{ ml}^{-1}$, which are at least three to seven orders of magnitude higher than previous reports based on plaque-forming units. This significant work indicated that viruses in the marine environment were most likely important controls of planktonic microorganisms as well as potential mediators of genetic exchange.

The discovery of high viral abundances in the ocean led to a wide variety of research into the impact of viral infection on marine planktonic organisms. Tailed bacteriophages have been described as the most abundant single type of organism in the biosphere (Hendrix, Hatfull, and Smith, 2003; Hendrix et al., 1999). Tailed viruses are common in the marine environment (Wommack and Colwell, 2000). Approximately 95% of all bacterial viruses, or bacteriophage, are tailed (Ackermann and DuBow, 1987). These combined observations, as well as the high abundance of bacterial hosts has led to the inference that most marine viruses are bacteriophages.

Ackermann (1998) suggested, based on current knowledge of their distribution and frequency, that tailed phages may have originated close to the origin of life 3.5 billion years ago.

(Ackermann, 1998) Since 1959 over 5,100 bacteriophages have been examined by TEM confirming the high prevalence of tailed bacteriophages, thus making them the largest viral group in nature (Ackermann, 2003).

Wommack and Colwell (2000) reviewed current research on marine viruses. Several recurring observations about marine viruses have come to be widely accepted. For example, in a wide variety of aquatic environments, even at great depths, viruses are generally present in about a ten to one greater abundance than the bacteria (Hara et al., 1996). Virus abundance in surface waters generally ranges from less than 10^4ml^{-1} to 10^8ml^{-1} , changing seasonally in conjunction with primary and secondary production.

Viral Impacts on the Microbial Loop

Viral infection has a distinct effect on microbial loop processes primarily due to their lysis of bacterial hosts, providing a significant source of DOM with nutrients needed for microbial loop cycling. Observation of natural populations of marine heterotrophic bacteria and cyanobacteria by TEM documented that up to 7% of the heterotrophic bacteria and 5% of the cyanobacteria from widely dispersed locations contained mature phage (Proctor and Fuhrman, 1990b). Visible, mature phages only occur in the latent period, which is about 10% of the total lytic cycle. Extrapolation of this figure to the entire lytic cycle suggests that 50-70% of heterotrophic and cyanobacteria, respectively are infected.

The discovery that viruses led to decreased overall primary productivity confirmed the importance of viruses in nutrient cycles in marine environments (Suttle, 1992; Suttle, Chan, and Cottrell, 1990). Based on independent studies, it has been estimated that up to 20% of marine bacteria are lysed daily, and that about 2-3% of primary productivity being lost to viral activity (Suttle, 1994; Weinbauer and Suttle, 1996a). The net effect of the “viral loop” within the microbial loop leads to a slight decrease in estimates for primary production and zooplankton production but an overall increase in bacterial production and respiration (Fuhrman, 1999).

Despite the many known viral forms and the high viral abundance observed in natural environments, most bacterial phyla are lacking described bacteriophages (Ackermann, 2003).

Viruses that have been isolated from medically or industrially important bacteria are the best characterized. Due to the anthropocentric focus of phage research to date, it is highly probable that many more environmental phages await discovery.

LYTIC VERSUS LYSOGENIC VIRAL LIFE CYCLES

Impacts of Lytic Viral Infections in the Marine Environment

Natural viral communities can cause infection and lysis of important marine planktonic communities, both heterotrophic and autotrophic. A large-scale mesocosm study compared the contribution of viruses and protists to the mortality of bacterioplankton (Fuhrman and Noble, 1995). About 75% of the total bacterial mortality was shown to be due to either viral lysis or consumption by protists, with both modes of mortality being about equal.

The abundance of viruses in aquatic environments can only be explained by continual cycles of infection and lysis. Fuhrman (1999) theoretically demonstrated that the maximum possible number of bacterial populations is equal to the number of unique resources plus the number of unique viral types. (Fuhrman, 1999) Even if viruses cause a relatively small proportion of mortality in a population, the number of viral types will have profound effects on community composition. Rapid viral propagation occurs when there is a high host density, which prevents domination of the population by one heterotrophic bacterial species (Fuhrman and Suttle, 1993). This same trend was observed in phytoplankton, with viruses exerting species control over the eukaryotic phytoplankton *Micromonas pusilla* (Cottrell and Suttle, 1991).

Modeling experiments have demonstrated a reciprocal relationship between bacterial diversity and viruses (Thingstad, 2000). This model was able to reproduce published observations and provide an explanation for coexistence of competing bacterial species. Because viruses tend to “kill the winner,” less dominant bacterial species can survive, leading to increased community diversity. Under this model, lysogeny acts as a repository, which prevents viral extinction owing to low viral abundance.

Viruses may also exert strong selection pressures on host evolution, specifically the evolution of host surface proteins, which are frequent targets of phages. Recent techniques developed for analysis of mutation rates within single microbial genomes has indicated that most genes for surface antigens in the bacteria examined were under strong selective pressure for amino acid substitutions (Plotkin, Dushoff, and Fraser, 2004).

Early research on the ecological impacts of viruses specific to phytoplankton demonstrated that seawater viral concentrates inhibited primary productivity, presumably by viral lysis of photosynthetic hosts (Suttle, 1992; Suttle, Chan, and Cottrell, 1990). These studies also reported that the phytoplankton recovered after several days, suggesting either growth of resistant hosts or development of resistance to the viruses, which is a common finding in all microbes.

Differing planktonic autotrophs express varying levels of susceptibility to viral infection. For example, natural *Synechococcus* communities demonstrated high abundances despite very high titers of lytic cyanophages infective for similar strains of *Synechococcus* (Suttle and Chan, 1994b; Waterbury and Valois, 1993). Marine cyanobacteria have demonstrated a relatively low rate of visible infection. Viruses have been calculated to account for only about 5-15% of the mortality of cyanobacteria, although a wide range of estimates can be cited, depending on the experimental method utilized (Fuhrman, 1999; Fuhrman and Suttle, 1993). Because of the low viral mortality in conjunction with high cyanophage abundances observed in marine environments, these researchers surmised that cyanophages have more profound effects on community composition than overall cyanobacterial abundance.

Ecological Importance of Algal Virus Infections

The discovery of infectious algal viruses lagged behind the discovery of heterotrophic bacteriophages by about fifty years. The search for freshwater algal viruses was heavily pursued during the 1960's and 70's because they were considered ideal candidates for development as algicides (Brown Jr., 1972; Suttle, 2000). Much research was concentrated on cyanophages,

which infect the ubiquitous cyanobacteria. After it became clear that cyanophages were not amenable to use as control agents, these investigations were abandoned.

A great deal of new research was focused on marine viruses after the discovery of profuse numbers of viruses in the ocean. Shortly afterward, a high abundance of viruses infecting marine cyanobacteria was revealed. Since that time, a great deal of research has focused on marine cyanophages due to the global ecological significance of their hosts.

Cyanophages cannot be enumerated directly by microscopy. They are quantified as a subset of the total marine viral community by plaque assay or by performing a dilution series of natural seawater, addition of a susceptible host, and quantification using a most probable number program (MPN). The MPN technique has been widely used for many algal types. The technique is limited by the fact that it detects only infective viruses and further, only those infective for the specific host used for enumeration. Even when more than one host strain is used for detection, it cannot be determined whether the phage titers are additive or overlapping. Despite these limitations, this method has demonstrated a similar level of precision to microscopy and plaque assay techniques (Cottrell and Suttle, 1995).

Cyanophages are an abundant component of natural seawater and are readily isolated from most marine environments. Their concentration measured by the MPN method generally varies from 10^2 ml⁻¹ of seawater to around 10^5 ml⁻¹, generally increasing with temperature, host abundance and salinity (Lu, Chen, and Hodson, 2001; Suttle and Chan, 1994b). The highest reported abundance of *Synechococcus* phages by this method is 10^6 ml⁻¹ (Mann, 2003).

Algal viruses can affect community composition, similarly to viruses of heterotrophic organisms (Tarutani, Nagasaki, and Yamaguchi, 2000). Cyanobacteria and their viruses co-evolve. Long term growth of *Plectonema boryanum*, a filamentous cyanobacterium, demonstrated that the host will develop resistance to the lytic viruses and rapid adaptation of the virus leads to a persistent low-grade viral infection with elimination of sensitive host organisms (Colishaw and Mersa, 1975) A similar phenomenon was also observed in the toxic bloom-forming species *Heterosigma akashiwo* (Tarutani, Nagasaki, and Yamaguchi, 2000).

Similarly to viruses infecting aquatic heterotrophic bacteria, ultraviolet radiation (UV) appears to be the main mechanism of deactivation of cyanophage (Suttle et al., 1993). However adsorption to particles and colloids in seawater may play a role as well (Noble and Fuhrman, 1997). Research has demonstrated that natural cyanophage communities tend to be more UV resistant in the summer than the spring or winter, while laboratory cyanophage isolates from the same community do not exhibit this pattern (Garza and Suttle, 1998). Cyanophage isolates tested were more UV resistant than heterotrophic bacteriophage isolates and that natural viral communities were more resistant than laboratory isolates (Weinbauer et al., 1999).

Another interesting difference between cyanophages and viruses of heterotrophic bacteria is the requirement for the energy produced by photophosphorylation by the photosynthetic host for viral replication [reviewed in (Suttle, 2000)]. Disruption of photosynthesis by inhibitors such as DCMU, CCCP, valinomycin, nigericin, or lack of CO₂ completely inhibited viral replication in a *Synechococcus* isolate (Sherman, 1976).

Taxonomy and Characteristics of Cyanophages

Lytic viruses have now been identified for numerous strains of both prokaryotic and eukaryotic algae. Many cyanobacterial phage-host systems have been isolated and described. Interestingly, the viruses infecting eukaryotic algae are almost exclusively of the polyhedral morphology (Van Etten, Lane, and Meints, 1991). However, viruses of the prokaryotic cyanobacteria, including *Synechococcus* and *Prochlorococcus*, have been observed to belong to one of the three well recognized bacteriophage families, Myoviridae, Siphoviridae or Podoviridae (Safferman et al., 1983). This general classification scheme for cyanophages remains in general use.

An icosahedral capsid, and a contractile tail that is separated from the capsid by a neck structure are characteristic of Myoviridae. Siphoviridae have a long non-contractile tail while Podoviridae have short non-contractile tails. Early characterization and comparisons of *Synechococcus* cyanophages demonstrated a typical genome size range for myoviruses of 80-85

kb and a slightly larger size range of 90-100 kb for siphoviruses (Wilson et al., 1993). An analysis of their structural proteins revealed similarities between phages of the same morphological class and clear differences between the two general types.

To date the large majority of lytic *Synechococcus* phages are the myovirus type, possibly because myoviruses may be easier to isolate than podo- and siphoviruses [reviewed in (Mann, 2003b)]. The host range of these phages is not tied to the geographical location of isolation and is highly variable between phage isolates. The cyanomyoviruses also tend to have a broad host range, with some strains able to infect cyanobacteria from their sister genus *Prochlorococcus*, suggesting a potential mechanism for gene transfer (Sullivan, Waterbury, and Chisholm, 2003). Although some cyanophages have a generally broad host range, marine cyanophages may not readily infect freshwater cyanobacteria (Suttle and Chan, 1993). In contrast, the cyanopodoviruses appear to be very host-specific, usually only infecting their host of isolation (Mann, 2003).

Definition and Prevalence of Lysogeny

Generally bacteriophages are considered an infective agent that both lyse and kill their host. However, some viruses are capable of forming a stable symbiosis with their host bacterium, which is called lysogeny in bacterial cells and transformation in animal and plant cells (Wagner and Hewlett, 2004). Viruses with this ability are termed temperate phages. These viruses adsorb to the host and inject their nucleic acid similarly to lytic viruses. However, after entry of the phage nucleic acid, the phage can follow one of two pathways. The phage can either enter a lytic cycle or become quiescent as a prophage, where it can integrate into the host genome or in some instances maintain itself as a plasmid (Fig. 1-3).

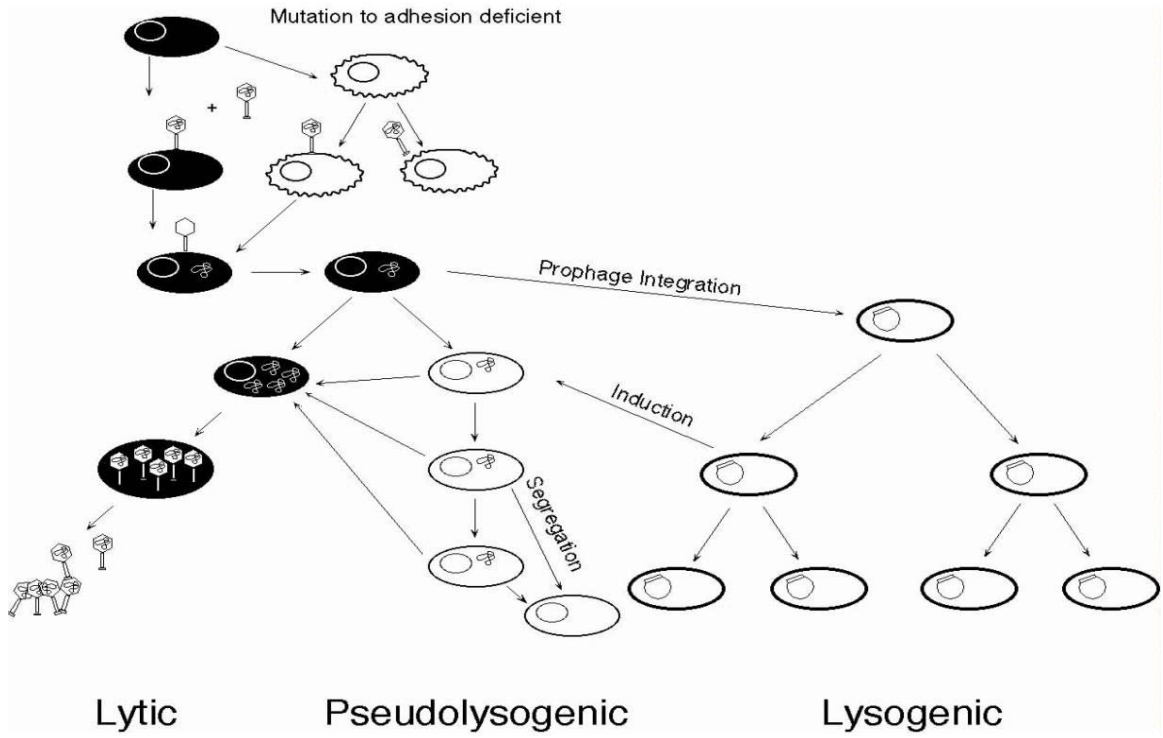


Figure 1-3: Illustration of lytic and lysogenic viral life cycles.

The insertion of viral DNA into the host chromosome is considered an ancient and important process. The genomes of many types of cellular organisms, both eukaryotic and prokaryotic, contain numerous functional and remnant viral chromosomes. For example, the human genome is estimated to contain about 8% retroviral DNA (Lander et al., 2001). In addition, some bacterial genomes have been demonstrated to contain up to 20% bacteriophage genes although these are not all necessarily functional prophages (Casjens et al., 2000).

The bifurcated life cycle used by temperate viruses is called the “lysogenic decision,” and has been studied extensively in the *Escherichia coli*/ λ system. In this system the decision is primarily based on the balance between two critical phage-encoded repressor proteins called *ci* and *cro*. The balance between these repressor proteins, thus the lysogenic decision, is generally determined by the metabolic status of the host organism.

When the host is healthy and undamaged, transcription of lytic viral genes is repressed, leading to entry into a lysogenic cycle with the phage remaining integrated at a specific site in the *E. coli* chromosome (Weaver, 1999). The *ci* protein encoded by the phage is the main repressor of lytic gene expression because of its control over viral promoter sites. The phage λ genome contains two promoters called P_L and P_R for left and right promoter. The P_L promoter controls lysogenic gene expression, while P_R controls lytic gene expression. The *ci* repressor protein binds to three operator regions adjacent to each promoter (O_{R1} , O_{R2} , O_{R3} , O_{L1} , O_{L2} , O_{L3}). Cooperative binding of *ci* to both operators 1 and 2 leads to repression of P_R and the lytic genes. The third operator site on the right side controls another weak promoter for the *ci* gene, which controls production of the *ci* protein itself. Therefore, when there is enough *ci* to bind the third operator site, the gene is repressed and no more *ci* is produced. Thus, it is an auto regulatory gene.

Recent research has also demonstrated that the repression of lytic genes requires the interaction of *ci* dimers on both the left and right operator sites. The protein dimers accomplish this by cooperative binding, causing a large hairpin loop in the DNA, leading to formation of octamers of *ci* (Dodd et al., 2001). The general promoter/operator configuration and formation of the stem-loop is illustrated in figure 1-4.

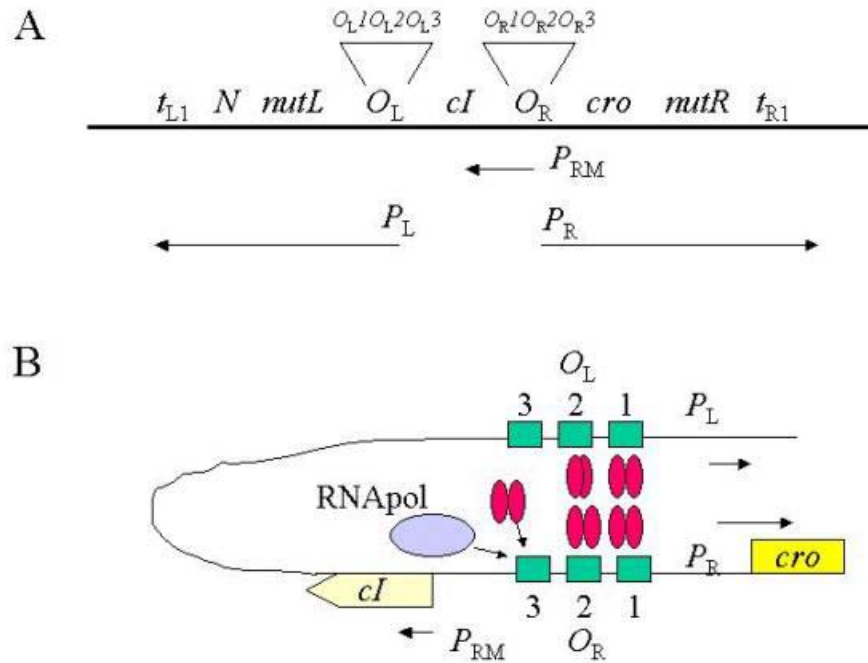


Figure 1-4: Panel A is a diagrammatic representation of the organization of the genes, promoters and operators controlling the “lysogenic switch” in phage λ . Panel B is a detailed illustration of the binding of CII dimers (red ovals) to the left and right operators and the formation of CII octamers. The cooperative binding of CII dimers leads to formation of a hairpin structure. This hairpin is required for effective repression of lytic phage genes.

When the host is stressed or subjected to DNA damage, the balance shifts due to enzymatic cleavage of the *ci* repressor by host-encoded *recA* protease. This cleavage of the repressor leads to excision of the prophage with initiation of a lytic cycle (Wagner and Hewlett, 2004). Similarly, under starvation conditions excision is induced by *HflA* protease, which is produced in response to increased cellular levels of cAMP.

Although mutagens such as UV light and Mitomycin C are the most commonly used inducing agents, other triggers of prophage induction have been identified. For example, for lysogenic strains of *Vibrio cholerae*, natural sunlight has been shown to be an effective trigger of prophage induction (Faruque et al., 2000). The induced prophages were biologically active, hinting at sunlight as a natural mechanism leading to generation of newly toxigenic strains of *V. cholerae*.

Although *E. coli* λ is one of the most well known lysogenic systems, not all phages utilize a similar mechanism. Three different metabolic strategies for the establishment of lysogeny have been described to date. The first, the λ type described above, is mediated by integrase insertion of the prophage into a specific site on the host chromosome with maintenance of the integrated prophage by a complex repressor system, which is subject to derepression with excision of the prophage. The second type is characterized by phage P-1 with the prophage persisting as a plasmid. The third type is typified by phage Mu, with random integration of the prophage mediated by a transposase (Ackermann, 2003; Ackermann and DuBow, 1987; Weaver, 1999).

Surprisingly, although lysogeny is often associated with *Siphoviridae*, it is not limited to tailed phages. It has been observed in some of the less well-known viral families such as *Fuselloviridae* and *Plasmaviridae* (pleomorphic, dsDNA), *Inoviridae* (filamentous, ssDNA) and *Lipothrixviridae* (filamentous, dsDNA) (Ackermann, 2003). The largely unexplored mechanisms of lysogeny used by these viral types include the use of integrases and host recombinases. This suggests the potential for totally novel mechanisms of lysogeny.

There was initially some debate about the importance of lysogeny in the marine environment (Wilcox and Fuhrman, 1994). A number of experiments appeared to indicate that lysogenic phage production was not an important source of phage production or bacterial

mortality, accounting for an estimated 0.14-8.8% of the total bacterial mortality (Weinbauer and Suttle, 1996; Weinbauer and Suttle, 1999). Although lysogeny did not have a large impact on viral abundance, similar research demonstrated a high prevalence of lysogeny does exist in certain environments (Cochran, Kellogg, and Paul, 1998; Jiang and Paul, 1996). These researchers also obtained and characterized lysogenic phage-host systems from natural heterotrophic marine bacteria (Jiang, Kellogg, and Paul, 1998).

Recently it has been revealed that lysogeny can be very important in both heterotrophic and autotrophic bacteria, but that it varies greatly both temporally and spatially (McDaniel et al., 2002). A study of the prevalence of lysogeny was performed over wide ranging ocean environments from surface to great depth and oxygenated to anoxic (Weinbauer, Brettar, and Höfle, 2003). It was demonstrated that lytic and lysogenic processes were related in the form of a negative power function, with lytic infections predominating in productive estuarine and offshore waters and lysogenic infections being more prominent in deep and anoxic areas.

Consequences of Lysogeny

Lysogeny has long been considered a survival strategy for viruses, allowing them to persist in environments with unfavorable conditions such as low host abundance. Modeling experiments predict that bacteriophages with low probabilities of lysogeny and low spontaneous induction rates (i.e., lytic phage) will be favored in equable environments, while bacteriophage with higher probabilities of lysogeny and higher spontaneous induction rates will be favored in more variable environments (Mittler, 1996; Thingstad, 2000). This paradigm has been supported by current research, which consistently demonstrates a correlation between the prevalence of lysogeny and conditions of low host abundance and low productivity (McDaniel et al., 2002; McDaniel and Paul, 2004; Weinbauer, Brettar, and Höfle, 2003).

When a temperate phage integrates as a prophage, it is not surprising that as a result there are some changes to host metabolism. One of the most common changes consists of changes to the protein composition of the host cell surface with a resulting change in immune signature (Wagner and Hewlett, 2004). This change in surface structure as well as production of

repressor proteins leads to bacterial resistance to similar viruses. This phenomenon is part of the host response to integration called homoimmunity and is generally considered a universal consequence of lysogeny.

The integrated prophage can both positively and negatively affect the fitness of its bacterial host in several respects [reviewed in (Brüssow, Canchaya, and Hardt, 2004)]. As mentioned previously, the fitness of the host can be improved by protection from infection by similar phages. The phage can also benefit the host by introduction of new host fitness factors or by lysis of competing strains through prophage induction. Less beneficial effects can occur through disruption of host genes by the insertion of a prophage. Prophage can also serve as anchor points for genome rearrangements, which may have positive, negative or neutral effects.

It is now known that temperate bacteriophages play a primary role in the development of pathogenesis in bacteria. Many of the genes for common bacterial toxins have been demonstrated to be prophage encoded [reviewed in (Brüssow, Canchaya, and Hardt, 2004)]. Some common extracellular bacterial toxins that are known to be prophage-encoded include: Diphtheria toxin, Neurotoxin, Shiga toxins, Enterohaemolysin, Cytotoxin, several Enterotoxins, Cholera toxin, Leukocidin and Cytolethal distending toxin. Phages can encode several other gene types that aid the bacteria in successfully infecting its host. Some examples are toxic enzymes, mitogenic factors, and effector proteins that assist in invasion. Besides production of toxins and other virulence factors, prophages cause pathogenicity by altering antigenicity of the host bacterium or by providing membrane proteins to aid in bacterial attachment to the host.

Providing virulence factors to the bacterial host improves its fitness, which is an advantage to the bacteria as well as the prophage. In addition, research has recently established that the production of phage-encoded toxins in pathogenic strains of *Staphylococcus aureus* is tied to the life cycle of the prophage, not to that of the host organism (Sumbly and Waldor, 2003).

A less well-known consequence of temperate bacteriophages is their capacity to confer spore-forming ability on the host. Spore-converting bacteriophages have most commonly been isolated for *Bacillus* and *Clostridium* species such as *B. subtilis*, *B. thuringiensis*, *B. pumilis* and *C. perfringens* (Silver-Mysliwiec and Bramucci, 1990). All of the known spore-forming

bacteriophages are pseudotemperate, where they form an unstable lysogenic relationship with their host organism.

Interestingly, the proteins regulating sporulation in *Bacillus subtilis* bear striking structural similarity to the well-known *E. coli* repressor proteins, CI and Cro (Lewis et al., 1998). The identical tertiary structure in the DNA binding domains of these protein complexes strongly suggests an evolutionary link between the processes of sporulation and prophage induction.

Effect of Mutagens

Mutagens are chemicals or conditions that cause direct damage to DNA. Common examples are polyaromatic hydrocarbons (PAH's), polychlorinated biphenyl's (PCB's), Mitomycin C and ultraviolet light (UV). These mutagens have long been observed to have profound effects on lysogenic phage-host systems. Most lysogens are detected experimentally by the ability of mutagens to cause increased production of viruses, termed prophage induction from a bacterial host.

In the heavily studied *Escherichia coli* / λ system, mutagens induce prophage by activation of a host DNA repair mechanism called the SOS response. This leads to synthesis of recA proteins, which cause cleavage of the CI repressors keeping the prophage quiescent. There are parallels in the general mechanisms of prophage induction in other lysogenic and latent (eukaryotic) viruses, however, the specific details are unique to each system (Wagner and Hewlett, 2004).

The potential practical utility of this phenomenon has been observed and employed in various ways. The earliest attempt to use prophage induction as an indicator for mutagens was performed using the *E. coli* / λ system (Thompson and Woods, 1975). Since then, lysogenic phage-host systems have been used for applications including the evaluation of the level of toxicity of drugs (Elespuru and White, 1983), exploring the protective effects of dietary antimutagens (Cabrera, 2000), and examination of environmental samples for potentially toxic contaminants (McDaniel et al., 2001; Vargas et al., 2001). A recent study has used prophage

induction as an indication of the potential toxic impact from the recreational use of sunscreen on the marine environment (Danovaro and Corinaldesi, 2003).

Lysogeny in Cyanobacteria

Lysogeny has been well documented in heterotrophic bacteria. However, there have only been fragmentary reports of lysogeny in autotrophic organisms. Some early reports documented the presence of lysogeny in the freshwater cyanobacterial strain *Plectonema boryanum* (Cannon, Shane, and Bush, 1971; Padan, Shilo, and Oppenheim, 1972). This cyanobacterium was inducible by the common inducing agent Mitomycin C as well as by elevated levels of heat (Rimon and Oppenheim, 1975). Similarly to lytic infection by *Synechococcus* cyanophages, photosynthesis was required for induction of cyanophages in lysogenic *Plectonema* isolates (Cochito and Goldstein, 1977).

A few other cyanobacterial types have been documented to have inducible prophage. For example, the marine filamentous, non-heterocystic cyanobacterium *Phormidium persicinum* was demonstrated to be inducible with Mitomycin C (Ohki and Fujita, 1996). The filamentous cyanobacterium *Trichodesmium*, which also plays an important role in marine environments as a major nitrogen-fixing organism, was verified to have inducible prophage (Ohki, 1999). Mitomycin C could stimulate prophage induction artificially and prophage induction was implicated as a probable mechanism of *Trichodesmium* bloom collapse.

Increases in viral abundances have been associated with collapses of many types of algal blooms (Bratbak, Egge, and Heldal, 1993; Drewes Milligan and Cosper, 1994; Tarutani, Nagasaki, and Yamaguchi, 2000). Recently, prophage induction of an algal virus was implicated in the collapse of a bloom of the toxic, filamentous cyanobacterium *Lyngbya majuscula* (Hewson, O'Neil, and Dennison, 2001). However, in this study the unique finding of cyanophage induction was inferred due to putative prophage induction observed in the UV treated bloom samples. Although this was one possible interpretation of the data, lytic infection cannot be ruled out.

Prophage induction in the unicellular *Synechococcus* has been reported only rarely. An inducible prophage has been described that infects the cyanobacterial strain NKBG 042902 (Sode et al., 1994). In this case, the phage was isolated from natural marine samples and was subsequently used to lysogenize a cultured marine *Synechococcus* host. Prophage induction occurred in the laboratory-infected host in response to UV light, Mitomycin C, and CuSO₄ (Sode, Onari, and Oozeki, 1997b; Sode et al., 1994).

Lysogeny has recently been demonstrated conclusively by prophage induction in natural populations of *Synechococcus* (McDaniel et al., 2002; Ortmann, Lawrence, and Suttle, 2002). When observed over an annual cycle, cyanophage induction was observed to be more prevalent in the late winter to early spring and was correlated with the factors low host abundance, and low level of primary productivity (McDaniel et al., 2002).

One study also indicated that the molecular mechanisms of lysogeny might be similar in cyanobacteria and heterotrophic bacteria (Owtrim and Coleman, 1987). As mentioned earlier, in the well-known lysogenic system of *E. coli*/λ prophage induction can be precipitated by synthesis of a protein known as RecA, in response to direct DNA damage. A *recA* homolog was isolated from the cyanobacteria *Anabaena variabilis*, which was able to restore spontaneous induction when introduced into *E. coli recA* deficient mutants. The authors suggested this is indicative of a similar mechanism of lysogeny to *E. coli*. However, it may only indicate a similar response to DNA damage.

PHAGE METAGENOMICS

Mosaic Viral Genomes

The completion of multiple virus genomes has allowed a comparison among several widely divergent as well as similar viral types. Groups of closely related bacteriophages, such as phages from low G+C content gram-positive bacteria, were observed to contain groups or “modules” of functionally related genes such as a lysogeny module, lysis module and DNA replication module (Lucchini, Desiere, and Brüßow, 1999).

It has been observed that a phage or prophage sometimes shares homology with one group of phages for a group of genes, such as replication, yet will be similar to a completely different group for genes encoding a different function (Rohwer et al., 2000; Romero, López, and García, 2004). For example, comparisons of gene organization and protein homology have demonstrated the evolutionary connection between phages and prophages from a phylogenetically wide range of bacteria including *E. coli*, *Streptomyces*, *Haemophilus influenzae* and *Mycobacterium tuberculosis* (Hendrix et al., 1999).

These analyses have led to the convincing argument that phage and prophage are constructed as gene “mosaics” from transfer of genes from a global viral gene pool where viruses all have access, but not necessarily the same level of access (Hendrix et al., 1999). Due to the mosaic nature of phages and the lack of any universal anchoring gene such as 16S, an alternative classification method has been proposed that incorporates the entire genome of the phage in its relationships to other phages (Rohwer and Edwards, 2002). This type of classification scheme is compatible with the currently used ICTV system but will allow more accurate classification into viral families. At this time the tree is heavily dominated by *E. coli* phage. Thus, the general topology of this viral tree will likely undergo major changes with the addition of more diverse viral genomes.

Evidence is accumulating that *Synechococcus* phages are also mosaic in nature. The first piece of evidence for this was the discovery of a homolog of a phage T4 portal protein gene (g20) in the cyanophage S-PM2 (Fuller, Wilson, and Mann, 1999). This gene exists in a cluster of viral structural genes, which is also very similar to phage T4 in gene order. Another example is the *Synechococcus* podovirus P60, which has been found to be very similar to phage T7, with a homologous DNA polymerase as well as overall gene synteny (Chen and Lu, 2002). In P60 there were also genes with similarity to other phages, including phage D29 from *Mycobacterium*. P60 also was found to contain putative cyanobacterial host genes for thymidylate synthase and some genetic components of ribonucleoside reductase genes. Similar ribonucleoside genes were also found in the genome of a marine roseophage S101 (Rohwer et al., 2000). These findings

suggest that cyanophages are subject to similar evolutionary processes, such as gene transfer, comparable to other well-studied phages.

Genetics of Lysogeny

The rapid pace of sequencing viral genomes has and continues to reveal that integrated viruses are common. A recent review of eighty-two completed bacterial genomes found that 51 contained readily recognizable prophages (Casjens, 2003). The proportion of prophage DNA contained within a specific bacterial genome can vary widely. The most extreme case to date is a pathogenic isolate of *E. coli*. (O157:H7), which contains 18 documented prophages accounting for 16% of its total genome (Canchaya et al., 2003).

The *in silico* recognition of prophages within genome sequences can be difficult. Integrated prophages are sometimes recognized by the presence of viral structural proteins, which are unique to viruses. Another method of identifying putative prophages is to search for integrases, which are often annotated in Gen Bank (Canchaya et al., 2003). There is a high probability that integrated prophages exist in greater abundance but they are not recognized due to both the astonishing array of viral diversity and the presence of less conclusive evidence such as partial or degraded viral chromosomes. In addition, hypothetical bacterial genes and even some definitive bacterial genes could be lysogenic conversion genes from unidentified prophages.

In addition to the fully functional inducible prophage, four additional types of prophage-related elements have been described: defective prophages (also called cryptic prophages), satellite prophages, bacteriocins and gene-transfer agents [reviewed in (Casjens, 2003b)]. Defective prophages are generally in a state of mutational decay but may still carry important functional metabolic or toxin producing genes. Satellite prophages are fully functional but lack structural genes and are depended on other phages for encapsidation. Some bacteria contain genes for proteins, which are not complete virions, yet resemble viral tails. These virus-like proteins are capable of killing bacteria, thus are termed bacteriocins. Gene-transfer agents are tailed virus-like particles that do not contain their own structural genes but carry random pieces of

bacterial DNA from host to host, where the shuttled DNA can recombine with a similar gene in its new host.

Many prophages operate by inserting their chromosome into the host's chromosome, commonly using site-specific recombination. The most common, although not the only known enzyme utilized for this purpose, is a form of integrase. These enzymes, also called recombinases, mediate the site-specific recombination between a viral (*attP*) and bacterial (*attB*) attachment site. Viral integrases have been described as falling into one of two general families, the tyrosine recombinases and the serine recombinases, based on their mode of catalysis (Groth and Calos, 2004).

Within the general group of tyrosine integrases, some researchers further subdivide the enzyme classification into those related to phage λ , and those related to phage P4 (Campbell, 2003). For both of these integrase types the insertion sites lie within tRNA genes, however the site within the gene differs. The lambdoid phages generally insert within the anticodon loops and the P4 phages insert downstream from this site in the T Ψ C loop. In addition, lambdoid phage integrases can evolve rapidly by frequent natural recombination with similar phages (Campbell, del Campillo-Campbell, and Ginsberg, 2002). As few as five non-contiguous amino acid changes have been demonstrated to change the integrase host-specificity

The common genetic arrangement of known temperate bacteriophages is illustrated by the *Streptococcus thermophilus* phage Φ O1205. This phage is characterized by a so-called "modular" gene arrangement, with the open reading frames (ORF's) involved in lysogeny clustered together and transcribed in the opposite orientation to the genes involved in lytic replication (Stanley et al., 1997). Similarly to *E. coli* phage λ , the phage contains an attachment site (*attP*), which is homologous to a bacterial attachment site (*attB*) and forms the specific position of phage integration by site-specific recombination. This phage also contains genes for an integrase/excisionase system analogous to phage λ (Fig. 1-5).

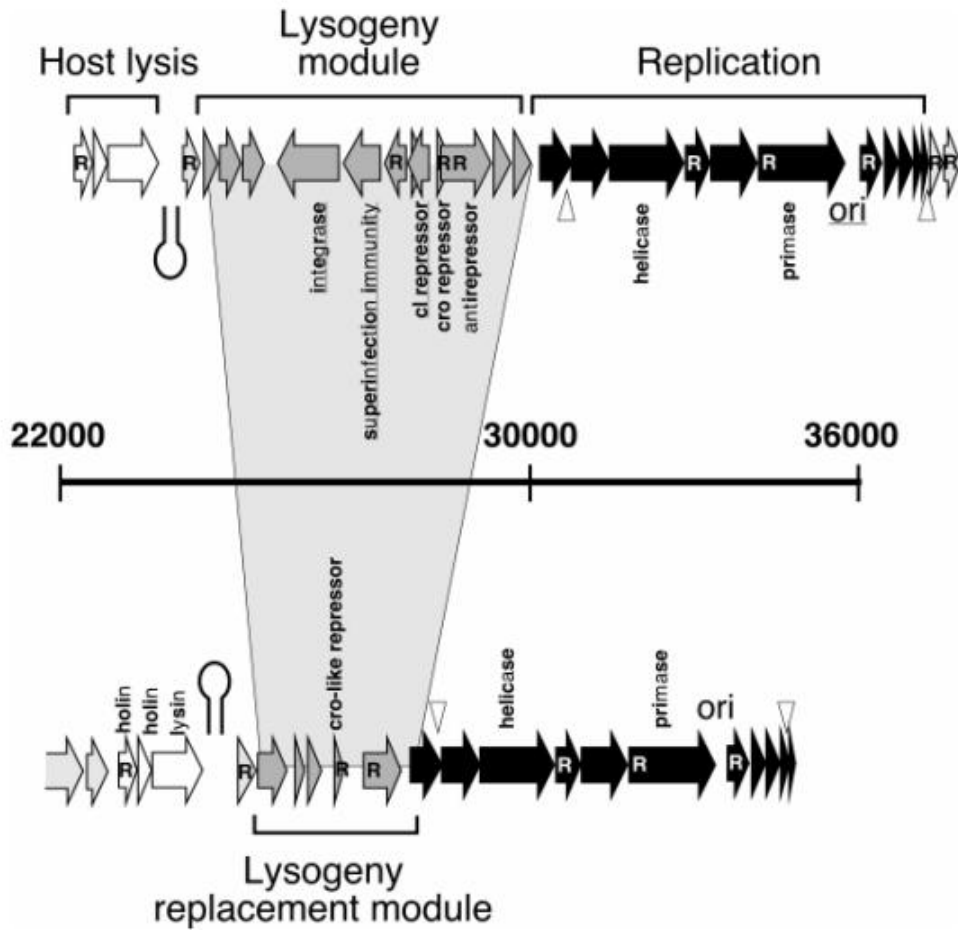


Figure 1-5: The top part of the figure demonstrates the typical arrangement of genes in a temperate phage. The genes involved in the lysogenic cycle are clustered together and in an opposite orientation to the lytic phage genes. The bottom figure denotes a lytic phage, in which the lytic genes are similar, however the lysogeny genes are partially deleted with the loss of temperate capability.

Closely related temperate phages contain their lysogeny genes in a nearly identical “module” of functionally related genes, with a specific type of lysogeny module able to define a viral group. This phenomenon was illustrated in a comparison of the lysogeny-related genes from fifteen temperate *Siphoviridae*, which revealed similarly organized lysogeny modules in eleven of the phages analyzed, all infecting hosts within the low G+C content gram-positive bacterial genera (Lucchini, Desiere, and Brüssow, 1999). Many lytic phages contain incomplete sets of lysogeny genes. This discovery has led to the conclusions that temperate viruses are the ancestral type (Madsen et al., 2001), and that lytic phages have resulted as a consequence of gene loss.

Viruses and Gene Transfer

Because prokaryotic organisms do not have any form of sexual reproduction, they evolve by either accumulation of mutations or by gene transfer with other organisms. Since entire genomes are never recombined, some genetic loci in prokaryotes tend to evolve more rapidly than others (Paul, 1999). Currently it appears that genes for basic metabolic functions such as replication, transcription and translation appear to be transferred infrequently (Ochman, Lawrence, and Grolsman, 2000). These so-called “hardware” genes contrast with “software” genes, which are transferred more readily.

There are three main mechanisms of prokaryotic gene transfer: conjugation, transformation, and transduction. Conjugation, also called bacterial mating, is the transfer of a genetic element called a plasmid between bacterial cells (Madigan, Martinko, and Parker, 2000). A plasmid is a genetic element that replicates independently of the host chromosome and may exist in variable copy numbers within the host cell. Unlike a virus, a plasmid exists inside the host cell as uncoated nucleic acid and does not have an extracellular form. Transfer of a plasmid is a complex process involving cell-to-cell contact via a pilus. An intricate gene pathway encoded by the plasmid itself mediates the process. Many different types of plasmids are known, encoding a variety of functions such as metabolic pathways, antibiotic resistance and virulence factors.

Transformation is the incorporation of free environmental DNA into a recipient cell, which can lead to genetic change. A bacterial cell that is able to accept molecules of DNA in this way is said to be competent. Some bacteria have the native ability to be transformable. However, the competent state can also be artificially induced in bacteria such as *E. coli*. The discovery of this process led to some of the major advances in the field of molecular biology (Madigan, Martinko, and Parker, 2000).

Transduction is the transfer of genetic material by a virus. However, viruses also indirectly lead to transformation by the release of free DNA as a consequence of viral lysis. The ability of viruses and bacteria to perform these functions has been well documented in the laboratory and widely used in molecular biology for construction of genetically engineered organisms (Lan and Reeves, 1996; Weaver, 1999).

Viral-mediated transduction can either be non-specific (generalized) or specific (specialized). Generalized transduction occurs by a packaging error during viral replication. Pieces of random plasmid or chromosomal DNA are placed in the viral capsid along with the viral genes. Specialized transduction is the inclusion of host genes flanking an integrated prophage during excision and initiation of a lytic cycle.

In environmental gene transfer events, transduction has some advantages over the other two types because the DNA is protected by the viral capsid and the virus can spread widely through the aquatic environment obviating the need for direct cell contact. This mode of gene transfer is possibly a means of transferring genetic material between widely divergent ecosystems. Sano et al (2004) documented that environmental viruses can successfully propagate in non-native ecosystems (Sano et al., 2004). In these experiments, both lake and sediment viral concentrates were able to successfully replicate on natural marine bacterial communities. These findings suggest the potential for viruses to transfer genetic material between disparate environments.

Gene transfer by transduction in the marine environment has been demonstrated experimentally (Jiang and Paul, 1998). Although the transduction frequencies were low, Jiang

and Paul (1998) used model estimates of the ecosystem being studied to calculate that potentially 1.3×10^{14} transduction events could occur per year in the Tampa Bay Estuary.

The accumulation of complete sequences from multiple phage genomes, along with the genomes of their hosts, has permitted bioinformatic discovery of putative gene transfer events. Many bacteriophages from widely divergent environments contain genes homologous to those found in their hosts. One example is the marine Roseophage SI01. This phage encodes four proteins utilized in phosphate metabolism that are homologous to genes found in the host organism that may have been acquired by gene transfer with the host (Rohwer et al., 2000).

Completed sequencing and analysis of the genome of the motile *Synechococcus* WH8102 indicated a strong influence of the genome from viral gene transfer (Palenik et al., 2003b). Sixteen probable or possible phage integrases, as well as remnants of phage integrase regulators, were identified within the WH8102 genome.

Not only can the host organism acquire genes from viruses, but also viruses may gain functional genes from its host. The genes for reaction center core proteins D-1 (gene *psbA*), D-2 (gene *psbD*) and a high light inducible protein, HLIP (gene *hli*) have recently been discovered on viruses infecting both *Synechococcus* and *Prochlorococcus* hosts (Lindell et al., 2004; Mann et al., 2003). Phylogenetic analysis supports the hypothesis that these genes are closely related between the viruses and the hosts they infect and that these genes have likely been transferred from host to phage multiple times.

Besides the obvious evolutionary consequences of gene transfer, prophages have provided a way for evolution of bacteria through major genome rearrangements, as well as gene duplication. The presence of integrated prophage has been demonstrated to allow gene inversion with strain differentiation in several bacteria including *Salmonella enterica* serovar Typhi, *S. pyogenes*, *Yersinia pestis* and the plant pathogen *Xylella fastidiosa* [reviewed in (Brüssow, Canchaya, and Hardt, 2004)].

Genomics of Marine Viruses

Despite the fact that a large number of bacteria and viruses have been sequenced, the main emphasis has been on isolates of commercial or medical importance. However, marine viruses play very important roles in carbon cycling through marine food webs as well as mediating gene transfer in aquatic environments causing host phenotype conversion by lysogeny (Paul and Sullivan, 2005). Only about 20 marine phage genomes have been sequenced as of this writing (Paul and Sullivan, 2005). Marine phages tend to have a lower level of homology to known viral types, highlighting the importance of the potential for discovery of novel genes and mechanisms from this viral group.

The most commonly sequenced marine phages to date appear to be vibriophages (6 of 20), possibly due to their ease of isolation and potential roles in pathogenicity. For example CTX Φ , the vibriophage known to be the causative agent of cholera, was the first marine phage to be sequenced and has been extensively studied (Davis et al., 2000; Waldor and Mekalanos, 1996). This temperate myovirus is known to carry the genes for the cholera toxin and is a prime example of the tendency of prophages to convert their bacterial host to a pathogenic phenotype. This phage is 33 kb in length, contains 44 open reading frames (ORF's), and is one of the few marine phages identified so far to carry recognizable lysogeny genes including an integrase and CI/CII-like repressors.

Two other marine vibriophages, which were originally isolated using *V. parahaemolyticus* st. 16 as host have been sequenced (Seguritan et al., 2003). These two phages initially appeared to be one phage isolate but were resolved into two closely related phages, one forming turbid plaques (VP16T, 45,575 bp) and one forming clear plaques (VP16C, 47,537 bp). These two marine vibriophages are identical across 62% of their genome and do appear to carry some genes possibly involved in pathogenicity. However, up to 75% of the genes from these two phages were not similar to any genes of known function.

The recently completed sequence of the marine bacteriophage *Listonella pelagia* Φ HSIC highlights the unique nature of marine phages. This phage was found to be approximately 38kbp in length with 47 open reading frames (ORF's). Only 20 of these ORF's produced significant

matches with sequences in GenBank. Of the twenty, only nine could be assigned a putative function: DNA polymerase, terminase, capsid and tail structural proteins, a lysozyme, a helicase, part of a resolvosome complex and a putative toxin/virulence gene (Paul et al., 2005). Although the virus exhibits a pseudolysogenic life cycle (Williamson, McLaughlin, and Paul, 2001), no lysogeny module or integrase were identifiable.

Genomics of Cyanophage

Only two complete marine cyanophage genomes have been published as of this writing but three additional genomes have been submitted for publication (Paul and Sullivan, 2005). The first, cyanophage P60 belongs to the genus *Podoviridae* and is an obligately lytic phage. This approximately 48 kbp phage was observed to contain 80 potential open reading frames with homology to previously described lytic phages ranging from 21-52% (Chen and Lu, 2002). These researchers observed that the DNA replication system of this podovirus was more highly conserved than similar siphoviruses and myoviruses, suggesting the potential importance of DNA replication in its life cycle. In addition, genes for nucleotide metabolism were similar to those from host organisms, suggesting potential gene transfer.

Despite the limited sampling to date, cyanophages have demonstrated some of the mosaic nature observed in phages of heterotrophic bacteria. The foremost example of this has been observed in genes encoding capsid structural proteins. The common T4 types of bacteriophages contain a conserved structural protein module (Tétart et al., 2001). A very similar module was recently discovered in marine cyanophages, indicating that some cyanophages have probably exchanged genes with the T4-like bacteriophages in the past (Hambly et al., 2001).

The presence of this capsid protein gene in cyanophages has been exploited to interrogate the diversity of natural cyanophage communities. A study of marine cyanophages from the oligotrophic Sargasso Sea to estuarine coastal environments revealed a high species richness of cyanophage, uncovering many unique types unrelated to known isolates (Zhong et al., 2002). The cyanophage communities were unique between dissimilar environments such as between the surface and the subsurface chlorophyll maximum (SCM).

In a similar study using primers based on the g20 gene, the diversity of cyanophages was observed in a Rhode Island coastal area over a period of three years (Marston and Sallee, 2003). During this study a typical seasonal pattern of high summer and low winter cyanophage abundance was observed, with the lowest number of genotypes during the winter months. Throughout the study 36 cyanophage genotypes were observed, ranging from one to nine distinct genotypes in each sample. New genotypes were discerned with each sampling, but a few genotypes continuously recurred. Importantly, the capsid protein gene could not be detected in up to 50% of isolated cyanophages, suggesting a limited utility of this gene in the study of natural cyanophage communities.

A more intriguing recent finding, based on the analysis of the second completely sequenced cyanophage genome (S-PM2), is the presence of core photosynthetic genes on cyanophages (Bailey et al., 2004; Lindell et al., 2004; Mann et al., 2003). Since discovery of these genes on S-PM2, eight cyanophages to date have been reported that contain the gene (*psbA*) for the photosynthetic reaction center protein D1 [reviewed in (Paul and Sullivan, 2005)]. As previously mentioned, active photosynthesis in the host is required for lytic production of cyanophages. Production of this rapidly degraded photosystem structural protein by the phage is likely to assist with a more efficient and/or prolonged production of viral particles.

All of the marine viruses sequenced to date are members of the three well-known dsDNA viral genera *Siphoviridae*, *Podoviridae* and *Myoviridae*. The recent description of lytic ssRNA viruses of phytoplankton highlights the narrow focus of marine viral research up to this point (Tai et al., 2003). It would be informative to investigate some of the less well-known viral groups in the marine environment such as *Leviviridae* or *Microviridae*.

SPECIFIC OBJECTIVES

Placed within the context of the reviewed literature, my objectives were as follows:

1. To develop a procedure for a MPIA to be used in screening marine samples for the presence of potentially carcinogenic pollutants.
2. To determine if lysogeny exists and is detectable in natural populations of *Synechococcus*.
3. To isolate cyanophage for investigation of host range and extract the DNA for investigation of cyanophage specific probes/primers.
4. To determine the proportion of *Synechococcus* isolates that are lysogenic based on the presence of prophage induction. To determine if there is a relationship between resistance to infection and whether or not an isolate is lysogenic.
5. To document the induced cyanophage using TEM.
6. To determine what physiologic and environmental cues catalyze the shift from temperate to lytic cycles in autotrophic prokaryotes, including nutrient limitation/stimulation, light regime, salinity and temperature.
7. To extract and sequence the viral DNA from a putative *Synechococcus* prophage and sequence the genome. The sequences will be interrogated for the presence of potential lysogeny genes and/or modules.

CHAPTER 2

Evaluation of Marine Bacterial Lysogens for Development of a Marine Prophage Induction Assay for Mutagen Detection in the Marine Environment.

The following chapter has been peer reviewed and published essentially in this form in the journal *Marine Biotechnology* (2001, Volume 3: p. 528-535)

CHAPTER SUMMARY

The demonstrated relationship between carcinogenicity of a chemical compound in mammals and its tendency to cause prophage induction in bacteria provides a method for biologically based carcinogen screening. Because of the need for this type of screening and the abundance of lysogens in the marine environment, fourteen isolates were evaluated for the degree of prophage induction in exponentially growing cultures in the presence of a known mutagen ($0.5 \mu\text{g ml}^{-1}$ Mitomycin C). Assays were performed in both liquid culture and in microtiter plates. Virus-like particles (VLP's) were enumerated by epifluorescence microscopy after staining with SYBR-Gold. Two isolates designated P94-4B3 (identified as *Halomonas aquamarina*) and P94-4S3 (identified as *Pseudomonas aeruginosa*) were further evaluated for potential use. Because of the rapid growth, larger size of its VLP, and linear response to increasing dose of Mitomycin C, the *P. aeruginosa* st. P94-4S3 was determined to be a better candidate for the MPIA. The *Pseudomonas* isolate was then used in several experiments for the development and optimization of the MPIA procedure. Initial screenings of the MPIA were also performed with selected environmental pollutants.

INTRODUCTION

Many known mutagenic compounds and mixtures are found in the environment in concentrations high enough to be a health hazard (Cerna et al., 1996). There is an ever-increasing need for mutagenesis assays to screen samples containing such compounds for potential carcinogenicity (DeMarini, Brooks, and Parkes, 1990). Existing toxicological testing can and is used to detect specific hazardous substances in environmental samples including mutagens, but there are limitations with such an approach. Toxicological testing can detect many specific hazardous and/or carcinogenic compounds, however environmental pollutants tend to be complex mixtures with a biological activity that is not easily predicted by the chemical profile (DeMarini et al., 1989). With biological testing it is possible to detect the combined effects of compounds, unknown substances, degradation products, metabolites and other synergistic or antagonistic effects, which are difficult to predict by toxicological testing (Helma et al., 1996). Existing methods of biological carcinogen testing often require the use of live animals. These procedures are time consuming, expensive and controversial.

An alternative basis for biological testing has been developed based on the strong demonstrated relationship between mutagenicity in bacteria and carcinogenicity of a compound (Ames, Lee, and Durston, 1973). The Ames test (Ames, Lee, and Durston, 1973) and the Rec-assay using *Bacillus subtilis* (Kada, Tutikawa, and Sadaie, 1972) have been developed as bacterial-based mutagenicity assays, which are alternatives to carcinogenicity testing with animals. These tests are an improvement over animal based testing but still require lengthy incubations and complicated procedures. Additionally, a lack of correlation was demonstrated between carcinogenicity and positive Ames test in a screening performed with 133 known carcinogenic compounds (Rossman et al., 1991).

Using prophage induction as a biological endpoint in testing provides another alternative for biologically based carcinogen screening due to the close parallel that has been demonstrated between the mechanisms of carcinogenesis and prophage induction (Ho and Ho, 1979). The Microscreen Assay, originally termed the Inductest, utilizes this principle (Moreau, Bailone, and Devoret, 1976). This assay has proven to be a more sensitive detection method for carcinogens

than the Ames test (Rossman et al., 1991). The Microscreen Assay utilizes the *Escherichia coli* λ phage system and is used for many environmental applications. However, this assay may not be directly compatible with marine samples (water and sediments), due to the optimal salinity range for the growth of *E. coli*. The goal of this study is to develop a Marine Prophage Induction Assay (MPIA) similar in concept to the Microscreen Assay, for detection of mutagens in the marine environment using a marine temperate phage-host system.

The Microscreen Assay utilizes the agar overlay technique to enumerate the number of plaque forming units (PFU's) as a means to estimate the level of prophage induction. This technique has been demonstrated to be accurate but has some disadvantages including that it is both time consuming and requires an additional non-lysogenic host. Another disadvantage of this technique seen during performance of the Microscreen Assay was lack of reproducibility. This was thought to be due to the toxic effect of the putative carcinogen on the viral particles, rendering some of the liberated viral particles as non-infective (DeMarini and Brooks, 1992). For these reasons the MPIA was developed using the techniques of SYBR staining and epifluorescence microscopy to ascertain the number of virus-like particles (VLP's) ml^{-1} directly.

MATERIALS AND METHODS

Isolation and Cultivation

Bacterial isolates were obtained from several offshore stations in the Gulf of Mexico during the summer of 1994 in the area of 24-28° N latitude and 82-86° W longitudes. These strains were isolated by the standard technique of plate streaking and single colony isolation on artificial salt-water media supplemented with peptone and yeast extracts [ASWJP; (Paul, 1982)]. After isolation the cultures were mixed 1:1 with sterile glycerin and stored at -40° C.

Screening of Isolates for Prophage Induction

Fourteen isolate cultures were incubated overnight in a shaker/incubator set at 28°C and 100 rpm. One ml of this culture served as an inoculum for 50ml of sterile medium and was incubated at 28°C with shaking at 100 rpm and growth monitored hourly by A_{600} . When the optical density of the culture reached an A_{600} of 0.4-0.6, the treatment flask was amended with Mitomycin C to a final concentration of 0.5 $\mu\text{g ml}^{-1}$. Samples for enumeration of viruses were taken at 3 hours and between 18 and 24 hours after treatment. Samples were centrifuged at 14,000x G for 12 minutes in an Eppendorf 5415C microcentrifuge to remove excess bacteria and the samples were fixed with 0.2 μm filtered formalin (1% final concentration).

The virus-like particles (VLP's) were enumerated using SYBR Green or Gold nucleic acid stain (Molecular Probes, Eugene, OR) and epifluorescence microscopy as described by Noble and Fuhrman (1998). In brief, SYBR Gold was diluted to 1:10 of the manufacturer's supplied concentration with 0.02 μm filtered deionized water. For each sample to be enumerated a 2.5 μl aliquot of the 10% working solution was added to a 97.5 μl drop of 0.02 μm filtered, deionized water on the bottom of a sterile plastic Petri dish (final dilution 2.5×10^{-3}). Using a 25 mm glass filtration set-up, a fixed sample was filtered through a 0.02 μm Anodisc filter (Whatman), backed by 0.8 μm cellulose mixed ester membrane (Millipore type AA). The Anodisc filter was filtered to dryness, removed with forceps with the vacuum still on, and placed sample side up on the prepared drop of stain in the dark and timed for 12 minutes. After the staining the filters were retrieved with a forceps, the back blotted with a Kimwipe and placed on a glass slide. A 36 μl drop of antifade solution was placed on the cover slip and inverted over the filter. All slides were enumerated using epifluorescence microscopy on an Olympus BX-60 epifluorescence microscope using 100x objectives and blue excitation (Noble and Fuhrman, 1998).

In performing the Microscreen Assay a result is scored as positive if the treatment value represents a threefold increase over the control (DeMarini, Brooks, and Parkes, 1990). Our criterion for selection of lysogens for further study was abundance of VLP's of approximately an order of magnitude greater than control. After initial screening the two isolates selected for

further experimentation were sent to MIDI labs (125 Sandy Drive; Newark, Delaware 19713) for identification by full-length 16S rDNA sequencing.

MPIA Development

For 15 ml tube assay, 105 ml of culture, prepared as previously described was divided and added to nine 15 ml Sarstedt tubes. Mitomycin C was added to the tubes for concentrations ranging from 0-0.5 $\mu\text{g ml}^{-1}$. These tubes were placed in a shaker-incubator at 28°C for an 8-hour incubation. VLP's were enumerated before and after the incubation by the SYBR technique.

For the nine-well plate assay, the isolate was grown as previously described by inoculation of stock culture into media and monitoring the A_{600} readings on an hourly basis. Two and one half ml of the prepared culture was placed in each well of a 9-well tissue culture plate (Costar; Cambridge, MA). Treatment samples were exposed to 0.5 $\mu\text{g ml}^{-1}$ concentration of Mitomycin C and the control cultures were exposed to an equal volume of sterile DI water. The lids were replaced, the seams sealed with parafilm, and the plates were then incubated on a gently circling orbital shaker at 28° C. Two duplicate sets of plates were prepared for sampling at 3 and 24 hours. VLP's were enumerated by SYBR Gold technique.

For the microtiter plate assay, 96-well microtiter plates (Corning; Corning, NY) were prepared by placing 50 μl of media in columns 2-6 and column 12 (control). Fifty-five μl of 2.5 $\mu\text{g ml}^{-1}$ Mitomycin C was placed in column 1. Five μl was transferred from column 1 to column 2 and mixed by trituration. This 1:10 dilution was continued through column 6. Two hundred μl of the actively growing culture was placed in each well for final Mitomycin C concentrations of 0-0.5 $\mu\text{g ml}^{-1}$. The lid was replaced on the plate and the edges sealed with parafilm. The plate was placed overnight on a gently circling orbital shaker in an incubator set at 28° C. All samples for enumeration of VLP's were fixed with filtered formalin for a final concentration of 1% and centrifuged to remove excess bacteria. SYBR Gold staining followed by epifluorescence microscopy was used for enumeration of viruses (Noble and Fuhrman, 1998). The general protocol is illustrated in Figure 2-1.

Protocol for MPIA

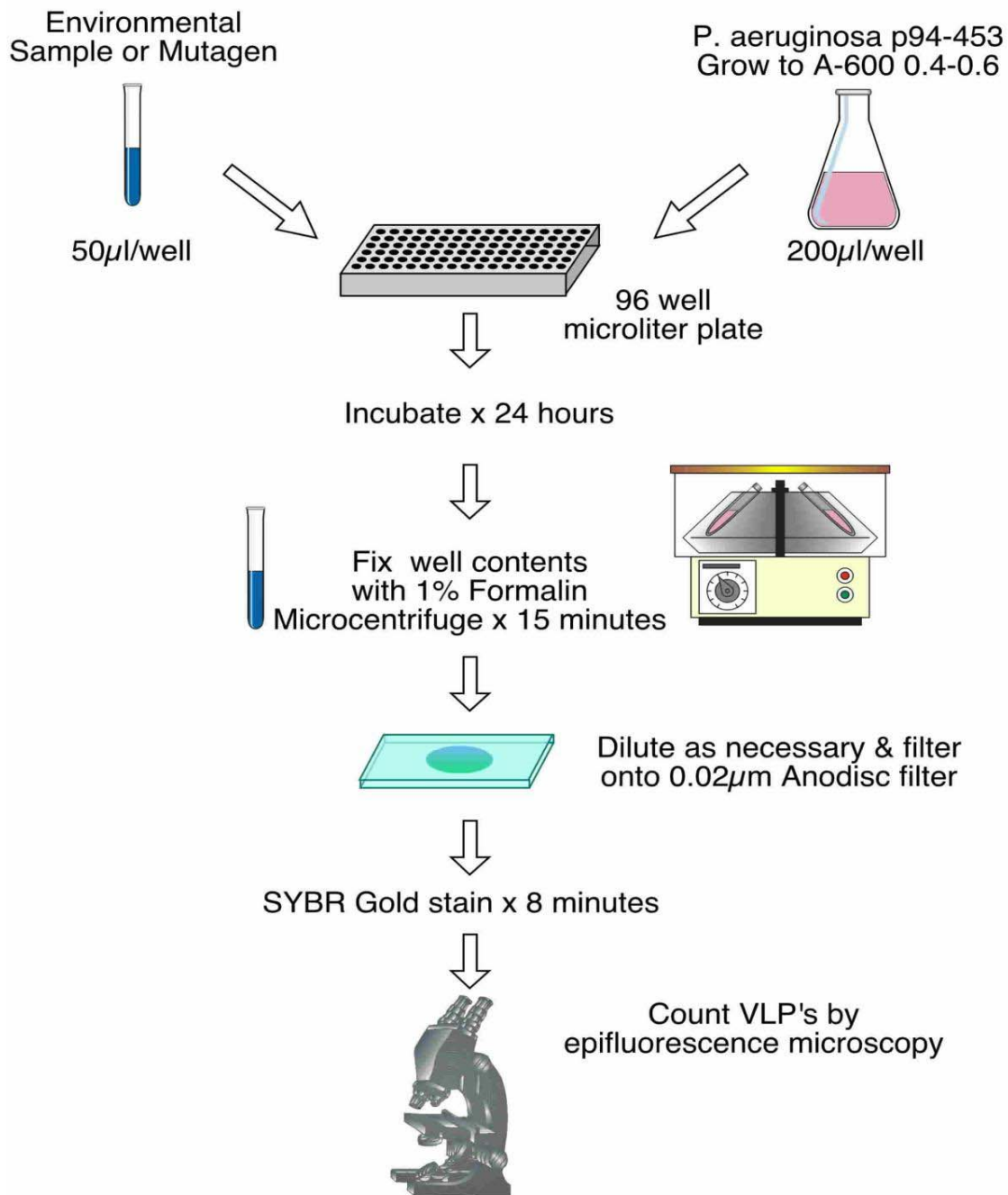


Figure 2-1: Illustration of the general protocol for the MPIA.

Pollutant screening by MPIA

Aldrin, malathion, naphthalene, phenanthrene and the polychlorinated biphenyl (PCB) Arochlor 1260 were screened for prophage induction by MPIA using the *P. aeruginosa* isolate. The concentrations of aldrin and naphthalene were 1 $\mu\text{g ml}^{-1}$ and 50 $\mu\text{g ml}^{-1}$ respectively, based on previous experiments of prophage induction using natural populations (Cochran, Kellogg, and Paul, 1998). Serial dilutions of malathion, phenanthrene and Arochlor 1260 were prepared using stock solutions to determine the sensitivity range of the isolate as described by DeMarini et al 1990.

The assay was performed using the microtiter plate procedure. The culture was prepared for the assay as previously described. The microtiter plate was prepared by diluting the selected agent in hexane and allowing the solvent in the loosely covered plate to evaporate overnight in the fume hood. The control wells were treated similarly with solvent only. Three hundred μl of culture in log-phase growth was placed in each well and the plate incubated overnight as previously described.

The assay was then repeated using a technique similar to one previously described for the Microscreen Assay with some modifications (Houk and DeMarini, 1988). The culture was prepared as previously described. A dilution series was prepared in a 96-well microtiter plate for each agent to be tested as well as a positive and negative control plate. Two hundred and fifty μl of medium was placed in the first column of wells. Fifty μl of the desired agent diluted in acetone, acetone (solvent control), Mitomycin C (positive control) or sterile DI water (negative control) was then added to the media in the first column and mixed by trituration. One hundred and fifty μl of media was then placed in all subsequent columns of wells. One hundred and fifty μl was transferred from the first column through column 12 and mixed well by trituration for a 1:2 dilution series. Seventy-five μl of the log-phase culture was then added to all wells for a total volume of 225 μl /well. The lids were replaced and all plates sealed with parafilm. The plates were placed on a gently circling orbital shaker at 28° C and allowed to incubate overnight.

In the morning the plates were observed for the amount of growth inhibition. The wells were observed to be slightly, moderately or fully turbid in comparison to the negative control. All wells were fixed with 1% formalin. Wells were sampled beginning with the moderately turbid wells. VLP's were enumerated by SYBR gold technique. All treatment counts were compared with a corresponding solvent control with the same percentage of acetone.

RESULTS

The criteria used in determining which isolate would be most useful for a Marine Prophage Induction Assay included production of phage in the presence of Mitomycin C in significant levels over control, large, easily counted phage, and an isolate that would grow consistently and quickly from stock culture.

Table 2-1 summarizes the results of the screening of isolates for prophage induction by Mitomycin C. Isolates designated as P94-4S3 and P94-4B3 showed the highest level of prophage induction of 14 isolates screened, yielding VLP abundances in the presence of Mitomycin C at least an order of magnitude over control. These two isolates were identified by 16S RNA sequencing as *Pseudomonas aeruginosa* (P94-4S3) and *Halomonas aquamarina* (P94-4B3). Both of these isolates exhibited rapid growth in liquid media (Fig. 2-1 and 2-2) reaching log phase within 3 hours and 5 hours, respectively. Phage production was easier to quantify with the *Pseudomonas* isolate as it produced larger, more visible phage. The *Halomonas* isolate produced greater numbers of VLP's during prophage induction in initial experiments but was not consistent in the level of prophage induction and was more difficult to enumerate due to small size of the phage and the tendency to produce background fluorescence with exposure to the SYBR stain. VLP production in the presence of Mitomycin C for initial 15 ml tube assay appears in Figures 2-1 and 2-2.

Table 2-1: Screening of Bacterial Isolates for Prophage Induction With Mitomycin C

Isolate Designation	Sampling Time	Treatment	Cell Density (A-600)	VLP's/ml	Observations
P94-7B4	18 hours	Control	1.423	7.5E+07	
		with Mitomycin C	0.337	5.0E+08	
P94-7B3	18 hours	Control	1.392	9.3E+07	
		with Mitomycin C	0.537	4.5E+08	
P94-7B2	18 hours	Control	1.515		unable to count
		with Mitomycin C	0.749		small virus
P94-7B1	18 hours	Control	1.427		unable to count
		with Mitomycin C	0.539		small virus
P94-5C5	18 hours	Control	0.866	9.8E+07	
		with Mitomycin C	0.3	6.7E+07	
P94-5C4	18 hours	Control	1.355	1.0E+08	
		with Mitomycin C	0.412	6.4E+07	
P94-4S3	18 hours	Control	1.524	5.0E+05	grows well
		with Mitomycin C	0.533	8.7E+06	large phage
P94-9D1	18 hours	Control	1.485	8.4E+07	
		with Mitomycin C	0.247	6.1E+07	
P94-4B3	24 hours	Control	1.071	1.5E+07	small phage
		with Mitomycin C	2.109	2.0E+11	background fluorescence
P94-4B4	24 hours	Control	1.83	0	No viruses detected
		with Mitomycin C	1.704	0	
P94-4S5	24 hours	Control	1.851	9.7E+09	
		with Mitomycin C	1.365	1.4E+10	
DO	24 hours	Control	1.939	8.8E+07	
		with Mitomycin C	1.591	1.5E+08	
P94-4S4	24 hours	Control	2.071	0	No viruses detected
		with Mitomycin C	1.684	0	
P94-4B5	24 hours	Control	2.239	0	No viruses detected
		with Mitomycin C	1.359	0	

Table 2-1: Initial screening of marine bacterial isolates by level of prophage induction for potential use in the MPIA.

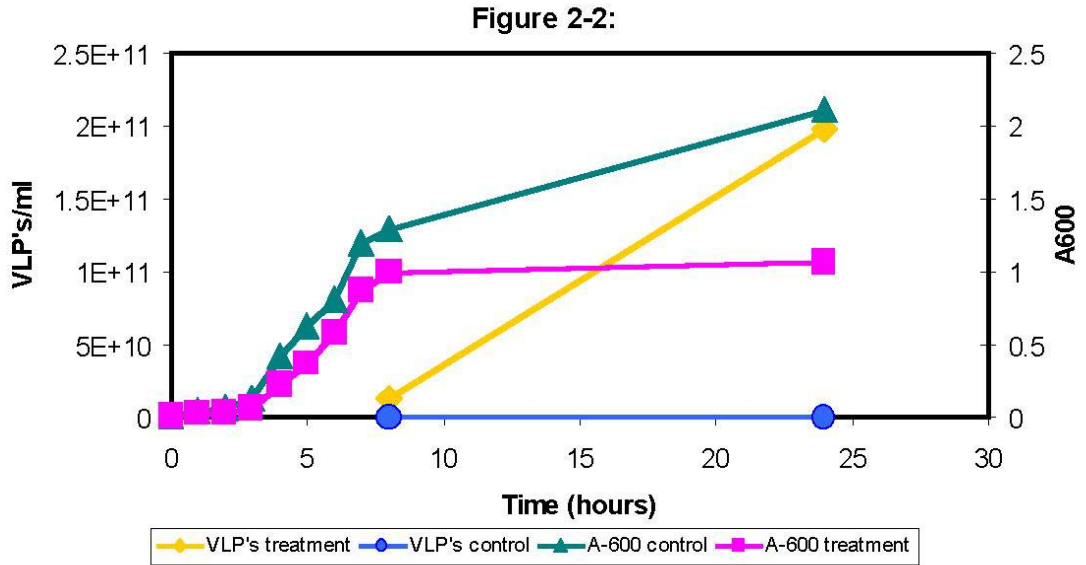


Figure 2-2: Screening of bacterial isolate P94-4B3 (*Halomonas aquamarina*) for growth and level of prophage induction using $0.5\mu\text{g ml}^{-1}$ of Mitomycin C. Growth rate monitored by A_{600} readings. The control culture growth is indicated by blue triangles and the treatment culture growth is indicated by the purple squares. Mitomycin C was added to the treatment culture when it reached log phase growth at about 8hrs. Note the large increase in VLP's in the treatment control in response to Mitomycin C.

Figure 2-3:

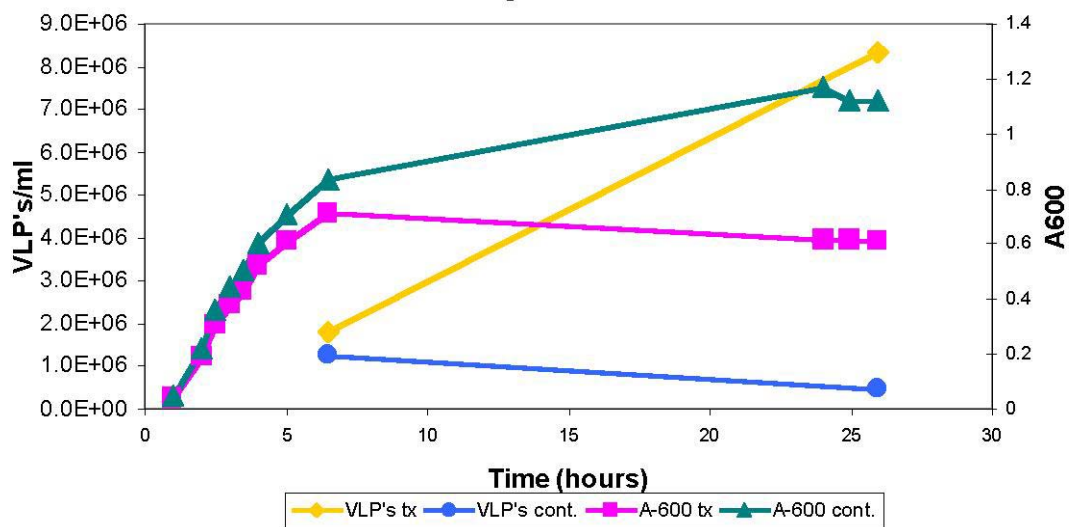


Figure 2-3: Screening of bacterial isolate P94-4S3 (*Pseudomonas aeruginosa*) for growth and prophage induction using 0.5 μ g ml⁻¹ of Mitomycin C. Growth rate monitored by A₆₀₀. Symbols are as indicated for figure 2-1.

The *Pseudomonas* isolate produced a maximum of 6.10×10^9 VLP's ml^{-1} compared to a maximum of 3.0×10^7 for the *Halomonas* isolate in a microtiter plate assay (Fig. 2-3). It is important to note that the *Pseudomonas* isolate demonstrated a linear response to the increasing dosage of Mitomycin C in the range from $0.1 \mu\text{g ml}^{-1}$ to 1 ng ml^{-1} . For these reasons the *Pseudomonas* isolate was selected for use in further development of the MPIA.

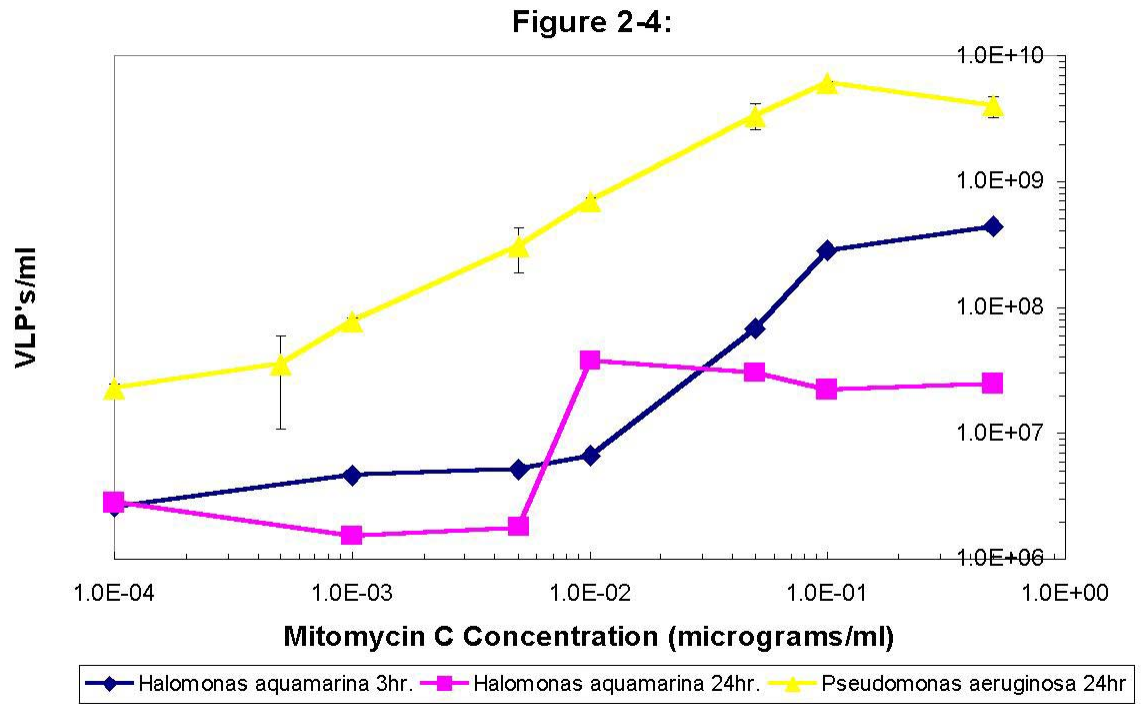


Figure 2-4: Comparison of the sensitivity of the *Halomonas* and *Pseudomonas* isolates to increasing concentrations of Mitomycin C in a microtiter MPIA.

The first step in optimizing the MPIA procedure was to determine the optimal culture volume and exposure time to the selected mutagen. The *P. aeruginosa* st. P94-4S3 was exposed to 0.5µg ml⁻¹ Mitomycin C in three different culture volumes and the level of prophage induction was monitored after 3 and 24-hour exposure times. The results are illustrated in Figure 2-4. The 24-hour exposure time clearly produced higher numbers of VLP's. The number of VLP's ml⁻¹ were similar for the intermediate and small volume experiments. The small volume microtiter plates were selected for further experimentation because they were more convenient and allowed a greater number of samples to be processed simultaneously.

Figure 2-5A: 3 Hour Incubation

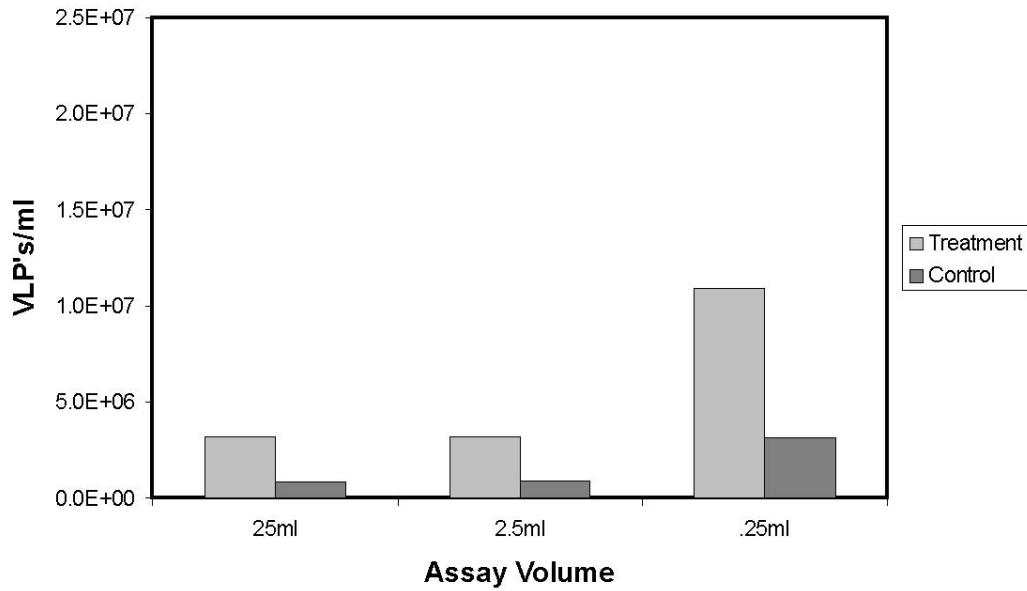


Figure 2-5B: 24 Hour Incubation

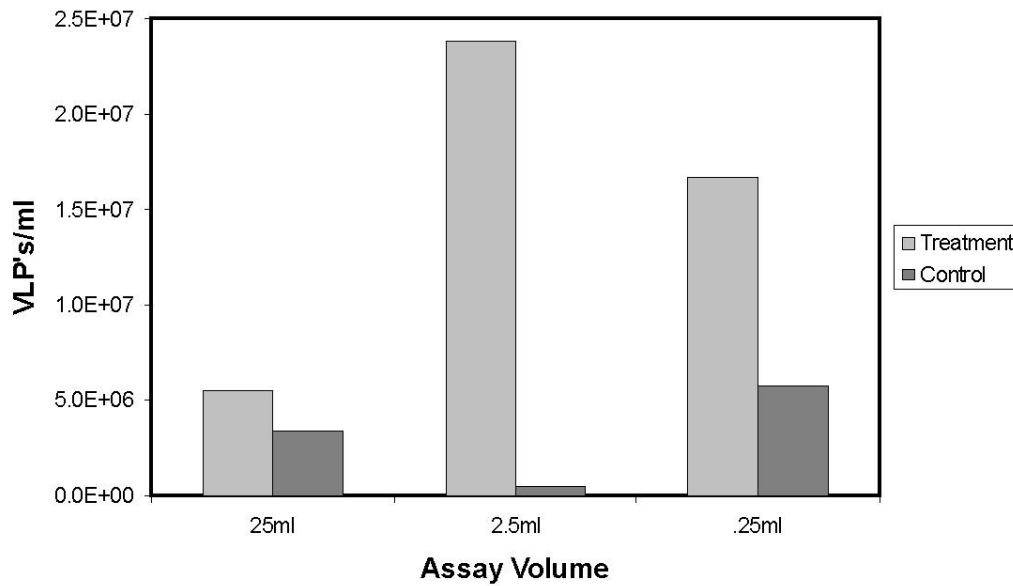


Figure 2-5: Comparison of level of prophage induction in the *Pseudomonas* isolate as a function of assay volume and time. The three-hour incubation is indicated by figure 2-4A, and the 24-hour incubation is indicated by figure 2-4B.

The modified microtiter MPIA using acetone as a solvent produced the clearest results in response to the environmental pollutants. The level of VLP's ml-1 was 588% of the control with the positive control, Mitomycin C. VLP's were 264% and 291% of the solvent control for naphthalene and Arochlor 1260 respectively. These results were statistically significant ($P=0.00732$ and $P=0.00196$). The pesticide aldrin produced VLP's ml-1 at 119% of the comparable solvent control. This was not found to be statistically significant ($P=0.232$).

DISCUSSION

Bacterial assays are becoming increasingly important in screening for genotoxic agents in the environment. The first reason for this is the strong correlation between the mutagenicity response in bacteria and mutagenic and tumor initiating activity in mammals; the second reason is the ease of performing this type of screening (Quillardet and Hofnung, 1988).

There are several bacterial screening tests that work by different physiological mechanisms. The specific physiological mechanism used for detection of mutagenesis is known as the endpoint of an assay (Rossman et al., 1991). For example, the Ames test detects mutagens by reversion of a known mutation in histidine metabolism (Ames, Lee, and Durston, 1973). The Ames test has been used more than any other short-term assay for assessing the genotoxicity of wastes. However, the Ames test performs poorly in detection of chlorinated compounds, carcinogenic metals and solvents (Houk and DeMarini, 1988). Prophage induction as an endpoint for a bacterial assay has been shown to be more sensitive indicator for a wider variety of mutagens.

The Microscreen Assay is based on detection of prophage induction caused by activation of the SOS system in response to DNA damage. There are other types of tests responding by different physiological end points but the phage induction type of assay appears to be sensitive to more varied types of substances probably due to sensitivity to more types of DNA damage (Vargas et al., 1995). For this reason a prophage induction type of assay similar in concept to the Microscreen Assay was selected for development.

The first step in the development of the MPIA was obtaining marine bacterial isolates. These isolates were screened for the presence of prophage induction using the standard inducing agent Mitomycin C, which is a demonstrated, potent mutagen. A screening of fourteen marine bacterial isolates demonstrated the presence of inducible prophage in six of the fourteen isolates. Two temperate phage-host systems, identified as *Halomonas aquamarina* st. P94-4B3 and *Pseudomonas aeruginosa* st. P94-4S3 were found to be potentially useful in the development of an MPIA due to the high level of prophage produced in response to the exposure to Mitomycin C. As previously discussed, the *P. aeruginosa* isolate was determined to be the better candidate for the MPIA. Although *P. aeruginosa* is often thought of as a terrestrial organism, it is widely distributed in aqueous environments as well as in soil. *P. Aeruginosa* has been isolated from widely divergent ecological niches (Griffin et al., 2001). It has been demonstrated to be an ecologically common and important organism responsible for breakdown of plant and animal materials in oxic habitats (Long, MacDonald, and Cairncross, 1991).

Direct viral enumeration by microscopy was required due to the lack of non-lysogenic strains of the bacterial isolates to perform the plaque assay technique. Epifluorescence microscopy was chosen over Transmission Electron Microscopy (TEM) enumeration of VLP's because of the relative simplicity of the epifluorescence technique as well as the tendency of TEM techniques to give a consistently lower number of viral particles (Hennes and Suttle, 1995). Epifluorescence microscopy has been determined to be the preferred method for evaluation of viral abundance because of its accuracy, simplicity, and suitability for processing large numbers of samples (Hara, Terauchi, and Koike, 1991). The epifluorescence method is also widely available which was an important consideration if the assay is to be made easily accessible for many laboratories.

The agents for initial tests of the assay were chosen as representatives of the general chemical classes, pesticides, polyaromatic hydrocarbons (PAH's) and PCB's; all with potential mutagenic activity. Aldrin, malathion, naphthalene and phenanthrene are pollutants, which have been found in significant concentrations in Tampa Bay, Florida (Long, MacDonald, and Cairncross, 1991). In addition, the Microscreen Assay has confirmed the mutagenicity of

phenanthrene and Arochlor 1260 (Rossman et al., 1991). In our study the results of the first experiment were not statistically significant. This is most likely due to limited solubility of the test compounds in the water-based culture media. When the solvent evaporation technique was used the final concentration of the test compounds were probably quite low. The modified technique used stock concentrations of the selected test compounds dissolved in acetone. The compounds were thus placed in direct contact with the actively growing culture and a statistically significant increase in VLP's ml-1 was then observed in response to the test compounds.

In the Microscreen Assay, it has been observed that the doses of a compound that produce positive responses are those just below the level of cytotoxicity (DeMarini and Brooks, 1992). A similar response was observed in the MPIA, with the highest level of response at the last column of slight growth inhibition (a moderately turbid well adjacent to a very turbid well). This observation will be useful in further testing of environmental pollutants.

The *P. aeruginosa* MPIA shows promise as a rapid screening tool for mutagenic pollutants in the marine environment. Further experimentation will be required to determine the range of agents detected by this assay. The assay will also require testing with environmental samples known to contain mutagens. In further development of the MPIA it will be helpful to consider the steps used in development of the Microscreen Assay. Similar work with environmental applications of the Microscreen Assay has determined that in all cases it is most desirable to use unprocessed environmental samples (Vargas et al., 1995). Any chemical extraction procedure decreases the sensitivity of the assay. This is the protocol that will be used when testing of environmental samples begins.

Possible microbial contamination of samples will also need to be considered when doing field-testing of the assay (Houk and DeMarini, 1988). Pre-filtration of the test samples may need to be considered to eliminate bacterial contamination. If it is feasible, automated counting technology will also be incorporated into the procedure for the MPIA in the future to increase the speed and ease of performing the assay.

CHAPTER 3

Use of the Marine Prophage Induction Assay (MPIA) To Detect Environmental Mutagens.

The following chapter has been submitted essentially in this form to the journal *Estuaries* and is in the process of peer review

CHAPTER SUMMARY

The prophage induction assay provides a biologically based carcinogen-screening tool for environmental samples grounded in the parallel mechanisms of carcinogenesis and prophage induction. Utilizing a previously developed assay with the characterized marine bacterial *Pseudomonas aeruginosa* isolate designated as P94-4S3 for the detection of potentially genotoxic contamination in marine and estuarine environments.

To perform the assay, the lysogenic isolate was exposed to either a known genotoxic compound or environmental sample of interest. The response was considered positive when a statistically significant amount of prophage induction occurred in comparison to negative controls. Initial development of the assay for environmental samples included testing under a range of salinities and optimizing the method for the processing of water column and sediment samples. The assay has been field-tested over two years in the Rookery Bay National Estuarine Research Reserve.

The Marine Prophage Induction Assay (MPIA) was performed concurrently with laboratory toxicological analysis. There was good correspondence between positive MPIA results and detection of potentially toxic compounds by laboratory analysis. Nine positive laboratory detections of known toxic compounds in natural samples occurred in conjunction with positive MPIA results. Six laboratory detections of compounds that are not genotoxic were accompanied by a negative MPIA response. Only one detection of a known toxic compound by the analytical lab was not accompanied by a positive induction response. Nine positive induction responses

occurred without concurrent laboratory detection. This was likely due to the limited range of compounds included in the laboratory testing performed, although false positive assay results cannot be ruled out.

INTRODUCTION

Rookery Bay National Estuarine Research Reserve is an 110,000-acre marine wetland reserve on the southern edge of the growing metropolis of Naples, Florida, and adjacent to the Florida Everglades. In addition to providing nearby Naples residents with opportunities for excellent boating and fishing, the reserve functions as a much-needed haven of undisturbed estuarine habitat on the densely populated west coast of Florida. The reserve managers are actively pursuing restoration of altered ecosystems and are attempting to restore the natural sheet water flow to the estuary. A main concern of reserve managers continues to be potential inputs of anthropogenic compounds from multiple non-point sources including roads, river and canal drainage, and nearby urban development. A method of screening Reserve samples for potentially bioactive contaminants was considered a high priority.

Unfortunately, there are many toxic compounds released into the environment as a result of human activity. Mutagenic compounds are of the highest concern because of their potential to harm both humans and ecosystems in general. It has been observed that known mutagenic compounds and mixtures are found in both soil and aquatic environment in concentrations high enough to be a health hazard (Cerna et al., 1996). There is an ever-increasing need for mutagenesis assays to screen samples containing such compounds for potential carcinogenicity (DeMarini and Brooks, 1992).

Standard laboratory toxicological testing can be used to detect specific hazardous substances in environmental samples including mutagens, but there are limitations with such an approach. Toxicological testing detects only the specific agents assayed, requiring a prior knowledge of what compounds should be monitored. In addition, environmental pollutants tend to be complex mixtures with synergistic biological activities that are not easily predicted by chemical profiles (DeMarini et al., 1989). Biological testing enables detection of the combined effects of compounds, unknown substances, degradation products, metabolites and their synergistic or antagonistic effects, which are difficult to predict by toxicological testing (Helma et al., 1996). Such an approach can serve as a pre-screening or a routine method for

environmental monitoring. When a positive response occurs, more intensive toxicological analysis can serve to identify the causative agent and hopefully the source of the contamination.

Many of the commonly used biological tests were developed based on the strong demonstrated relationship between mutagenicity in bacteria and carcinogenicity of a compound (Ames, Lee, and Durston, 1973). The Ames test (Ames, Lee, and Durston, 1973) and the Rec-assay using *Bacillus subtilis* (Kada, Tutikawa, and Sadaie, 1972) were developed as bacterial-based reverse mutagenicity assays, which provided alternatives to carcinogenicity testing with animals. These tests are an improvement over in-vivo testing but still require lengthy incubations and complicated procedures. Additionally, a lack of correlation was demonstrated between carcinogenicity and a positive Ames test in a screening performed with 133 known carcinogenic compounds (Rossman et al., 1991).

The prophage induction assay is another alternative for biological based carcinogen screening due to the close parallel that has been demonstrated between the mechanisms of carcinogenesis and prophage induction (Ho and Ho, 1979). Prophage induction was initially identified as a superior biological basis for testing because it responds to a wide variety of antimetabolites, not just direct DNA damage (Elespuru and White, 1983). One of the earliest attempts to use prophage induction as an indicator for the detection of carcinogens was performed with the *E. coli*/λ system (Thompson and Woods, 1975).

The Microscreen Assay, originally termed the Inductest, also utilizes this principle (Moreau, Bailone, and Devoret, 1976). The Microscreen assay has proven to be a more sensitive detection method for carcinogens than the Ames test (Rossman et al., 1991). This assay utilizes the *Escherichia coli*/λ phage system and has been used to investigate the mutagenic potential of therapeutic agents (Akerlele and Obaseiki-Ebor, 2002; Cabrera, 2000). The assay has also been adapted for use in examination of river ecosystems (Vargas et al., 1995). However, this assay may not be directly compatible with marine samples (water and sediments), due to the optimal salinity range for the growth of *E. coli*. The MPIA, similar in concept to the Microscreen Assay was developed specifically for detection of mutagens in the marine environment using a marine temperate phage-host system (McDaniel et al., 2001). During initial testing the MPIA

demonstrated significant positive prophage induction response to several environmentally relevant pollutants including pesticides, polychlorinated biphenyls (PCB's) and polyaromatic hydrocarbons (PAH's).

Although the MPIA was developed similarly to the Microscreen Assay, there are some procedural differences. The Microscreen Assay utilizes the agar overlay technique to enumerate the number of plaque-forming units (PFU's) as a means to estimate the level of prophage induction (Adams, 1959). This technique is accurate, but it is both time consuming and requires an additional uninfected host. Another disadvantage of this technique is the lack of reproducibility during use in Microscreen Assays observed by others. This was thought to be due to the direct toxic effect of the putative carcinogen on the viral particles, rendering some of the liberated viral particles as non-infective (DeMarini and Brooks, 1992).

Another standard technique for viral enumeration is counting by transmission electron microscopy (TEM). This method has several drawbacks including expensive equipment; intensive time-consuming sample preparation, as well as the inability to use this technique for work in the field (Noble and Fuhrman, 1998). In addition, a comparison of TEM methods with newer techniques using epifluorescence microscopy showed that the TEM methods provided significantly lower precision than epifluorescence techniques (Bettarel et al., 2000).

Techniques for viral enumeration using epifluorescence microscopy include the use of various nucleic acid staining methods. Commonly used fluorochromes include DAPI, YOPRO-1, and SYBR Green I (Bettarel et al., 2000; Noble and Fuhrman, 1998). A newer SYBR stain termed SYBR Gold has been developed, which yields a much brighter longer-lasting fluorescence signal (Chen et al., 2001). Epifluorescence microscopy using the SYBR-Gold staining technique is ideal for enumerating viruses in the marine environment. The SYBR-Gold stain has been observed to stain both RNA and DNA viruses, but not detritus. For these reasons the MPIA was developed using epifluorescence microscopy and SYBR-Gold staining to directly enumerate the number of virus-like particles (VLP's) ml^{-1} .

The first goal of this study was to develop a protocol for performing the assay on natural samples and to calibrate the assay results using standard toxicological testing and a known

contaminated sampling site. The second goal of this study was to utilize the field trial and laboratory data to determine whether or not the MPIA would be a useful screening tool for environmental water column and sediment samples in an estuarine environment.

MATERIALS AND METHODS

Study area/Sampling Sites

This study was performed from August 2002 through April 2004 at the Rookery Bay National Estuarine Research Reserve in Naples, Florida (Fig. 3-1). Three sampling sites were selected; Lower Henderson Creek (Rookery Bay), Lower Blackwater River, and Fakahatchee Bay, Figure 3-1. Each of the three sites was selected based on level of impact by nearby development and the presence of existing water quality monitoring stations. These water quality stations had continuous water quality data monitors that were used in determining if existing water quality conditions had any impact on assay results. The Fakahatchee Bay site was considered the least impacted by ongoing development and therefore was considered a reference site. The Blackwater River site was considered mildly to moderately impacted by development and the Henderson Creek/Rookery Bay site was considered the most likely to be contaminated due to runoff from nearby roads and construction sites. The Naples Bay site, which is located next to a busy marina was considered a potentially contaminated site and was sampled once at the conclusion of the study.

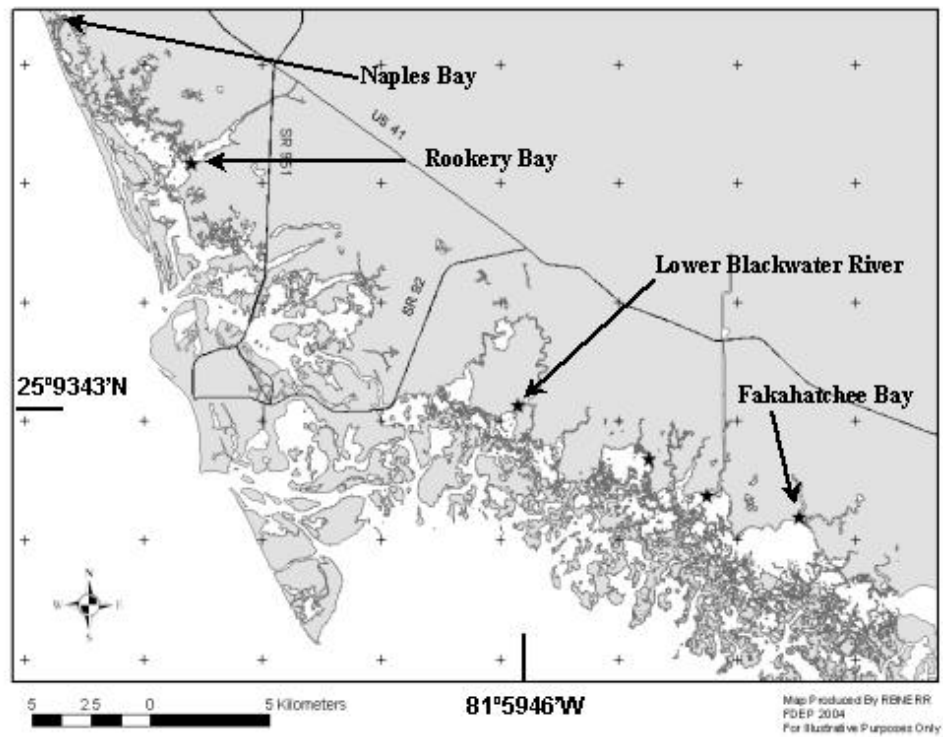


Figure 3-1: Reserve Map. Sampling sites for development of the MPIA were located at the Lower Henderson Creek (26°0257'N, 81°7332'W), Middle Blackwater River (25°9343N, 81°5946W), Fakahatchee Bay (25°8922'N, 81°4770'W), and Naples Bay (26°1298'N, 81°7921'W) water quality stations.

Environmental positive control sites, Sweetwater River and Bullfrog Creek (Fig. 3-2), were selected based on known heavy contamination from the previously conducted Tampa Bay Healthy Beaches study (Rose, Paul, and McLaughlin, 1999-2000). Both sites were found to have heavy levels of bacterial contamination from nearby wastewater treatment facilities and agriculture. Potential inputs of mutagenic compounds were also suspected.

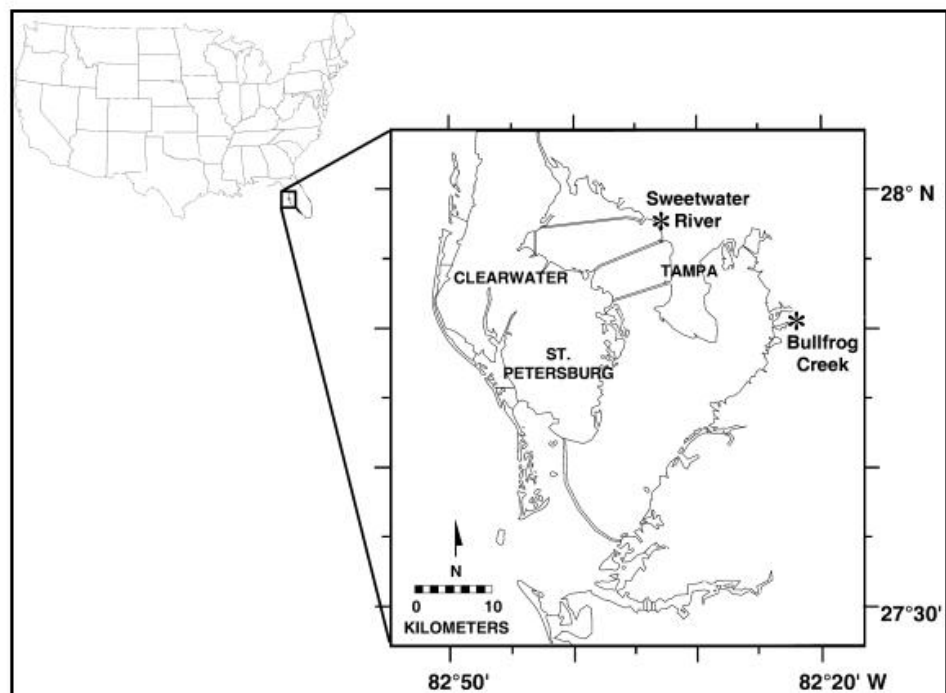


Figure 3-2: Map of known contaminated (positive control) sampling sites. The Bullfrog Creek and Sweetwater River sites are labeled with an asterisk.

MPIA Procedure

The MPIA was performed using a technique similar to one previously described for the Microscreen Assay with some modifications (DeMarini, Brooks, and Parkes, 1990; Houk and DeMarini, 1988). The *Pseudomonas aeruginosa* strain and the protocol used for the assay have been previously described (McDaniel et al., 2001). Briefly, in preparation for performing the assay, one colony of the bacteria was inoculated into one milliliter of growth media in triplicate and allowed to incubate overnight on a rotary shaker at 28° C. This culture was used to inoculate 30ml of growth media and returned to the shaker incubator. Growth of the cultures was monitored by A 600.

A 1:2 dilution series using 100 µl of the sample and 100 µl of bacterial growth media was prepared in a 96-well microtiter plate for each sample to be tested as well as a positive and negative control plate followed by the addition of 75 µl of the bacterial isolate in the logarithmic phase of growth. The positive control plate contained a dilution series of the potent mutagenic compound Mitomycin C. The negative controls consisted of a similarly prepared plate using sterile artificial seawater. The bacterial growth medium was sterile artificial saltwater supplemented with peptone and yeast extracts (Paul, 1982).

If the assay was performed on a known compound for verification of an experimental result, the plates were prepared similarly using an appropriate non-toxic solvent (i.e. acetone or methanol). In this case a negative control plate was also prepared similarly using only diluted solvent. All comparisons between treatment and controls were made between plates with the corresponding negative or solvent negative control with the same dilution factor or percentage of solvent (Houk and DeMarini, 1987).

Enumeration of Viruses

The virus-like particles (VLP's) were enumerated using SYBR Gold nucleic acid stain (Molecular Probes, Eugene, OR), with epifluorescence microscopy using an Olympus BX-60

epifluorescence microscope with 100x objectives and blue excitation essentially as described by Noble and Fuhrman (1998).

Procedures for Environmental Samples

The Rookery Bay Reserve has widely varying levels of salinity depending on the amount of freshwater input to differing sites. The assay was performed as described above utilizing control samples ranging from normal seawater salinity (35), decreasing to freshwater level (0). The level of VLP's was compared between samples using SYBR staining as above.

Adapting the assay protocol to the environment began with samples from the water column. The assay was performed utilizing the Rookery Bay Reserve seawater samples, which were pre-filtered either to remove natural bacteria (0.2 μm filtration) or to remove both bacteria and viruses (0.02 μm filtration) before being used in the assay. This step was completed to determine which method would give adequate sensitivity without interference from natural bacteria and viruses. The virus-free (0.02 μm filtration) technique was selected for use in all further MPIA testing.

Duplicate water samples were obtained simultaneously and sent on ice to the Florida Department of Environmental Protection (DEP) Central Laboratory for toxicological screening (2600 Blair Stone Road, Tallahassee, FL 32399-2400). The first three field samplings included testing for pesticides and heavy metals. Due to positive MPIA results without possible pollutant detection, the subsequent sample testing was expanded to assay for priority organic pollutants, including polychlorinated biphenyls (PCB's) and polyaromatic hydrocarbons (PAH's). Subsequent pesticide analysis included assays for organochlorine, organonitrogen, and phosphorus pesticides, and testing for herbicides. This expanded range was used for all subsequent water column and sediment samples.

The assay was adapted for use with sediment samples using a protocol similar to the previously performed study of river sediment samples utilizing the Microscreen Assay (Vargas et al., 2001). This prior study compared various solvent extraction methods on the sediments and

determined that the samples were best left untreated to prevent any loss of genotoxic compounds (Vargas et al., 1995).

Sediment samples were obtained at each site using a sediment push-corer. The first few centimeters of sediments were used to determine the most recently deposited contaminants. The samples were transported on ice to the laboratory and processed within 24 hours. Identical samples for toxicological screening were also placed on ice and sent with the corresponding water column samples to the DEP Laboratory for processing as previously described. The MPIA sediment samples were thoroughly mixed to homogenize the sediments and then centrifuged at 10,000x g for 10 minutes to extract the pore water. The pore water was 0.02 μm filtered and used in the MPIA as above.

Environmental contaminants that were detected by toxicological screening were verified as probable causes of a positive MPIA response by being tested individually in the assay using an appropriate dilution solvent (i.e. acetone or methanol), which that had previously demonstrated to be non-toxic to the bacterial isolate used for the assay.

Statistical Analyses

Two general types of statistical analysis were conducted. First, control viral counts and MPIA sample counts were evaluated by paired t-test between samples, and replicate Naples Bay samples were compared by ANOVA using Minitab statistical software. In addition to standard statistical testing, multivariate analysis of MPIA induction parameters and measured environmental parameters were conducted using Primer v.5.2.9 software (Primer-E Ltd., Plymouth Marine Laboratory, U.K. www.primer-e.com). The similarity matrices of both the induction parameters and water quality parameters were constructed utilizing normalized Euclidian distances. These matrices were compared to determine if a statistically significant relationship existed between them utilizing the RELATE test (sample statistic ρ).

Water Quality Data

General water quality indicators including temperature, salinity, dissolved oxygen, depth, pH and turbidity, were obtained from YSI model 6600 extended deployment system (EDS) data sondes permanently moored at the Reserve's water quality stations. The data was collected continuously and directly downloaded from the data-sonde to a personal computer for outlier analysis by the reserve staff. Data from the sites that corresponded to the time points for the MPIA sampling were utilized in the statistical analysis (Table 3-1).

Water Quality Parameters:								
Fakahatchee Bay								
Date m/d/y	Temp C	SpCond mS/cm	Sal	DOsat %	DO mg/L	Depth meters	pH	Turb NTU
8/2/2002	34.2	25.88	15.62	86.4	5.6	0.231	7.75	8.9
10/25/2002	29.32	29.89	18.42	57.4	3.97	0.222	7.58	81.3
12/19/2002	21.05	31.13	19.4	112.4	8.94	0.546	8.06	14.3
1/23/2003								
3/20/2003	28.62	50.19	32.78	85.8	5.54	0.463	7.55	24.5
5/6/2003								
6/24/2003	28.5	29.09	17.89	31.1	2.18	1.177	7.5	8.7
8/11/2003	29.41	19.8	11.73	59.7	4.27	1.195	7.67	17
10/13/2003	29.63	11.7	6.62	44.9	3.29	0.374	7.49	7.5
11/10/2003	26.96	12.82	7.34	68.4	5.23	0.302	7.5	10.3
Middle Blackwater River								
Date m/d/y	Temp C	SpCond mS/cm	Sal	DOsat %	DO mg/L	Depth meters	pH	Turb NTU
8/5/2002	31.94	41.55	26.44	56	3.55	0.762	7.73	9
10/25/2002	29.38	39.1	24.78	48.3	3.22	0.352	7.33	3.3
12/19/2002	19.21	37.71	23.98	66.1	5.3	0.332	7.54	9.2
1/23/2003								
3/20/2002	27.91	52.78	34.71	58.1	3.76	0.355	7.4	20.9
5/6/2003								
6/24/2003	27.97	41.37	26.42	26.6	1.8	1.303	7.23	8.6
8/11/2003	28.52	24.24	14.64	21.7	1.55	1.203		
10/13/2003	28.1	5.78	3.11			0.454	7.18	5.7
11/10/2003	25.96	9.628	5.4	35.7	2.81	0.357	7.24	7.6
Lower Henderson Creek (Rookery Bay)								
Date m/d/y	Temp C	SpCond mS/cm	Sal	DOsat %	DO mg/L	Depth meters	pH	Turb NTU
8/5/2002	32.85	42.72	27.24	81.3	5.05	1.054	7.88	29
10/25/2002	28.54	46.94	30.41	39.5	2.59	0.043	7.59	7.3
12/19/2002	18.97	43.29	27.94	81	6.37	0.63	7.95	7.3
1/23/2003								
3/20/2003	27.67	52.2	34.29	56.3	3.66	0.896	7.55	
5/6/2003								
6/23/2003	28.64	43.32	27.8			1.495	7.64	4.5
7/22/2003	32.09	46.49	29.97	63.6	3.94	1.447	7.79	6.5
10/13/2003	29.95	37.11	23.37	52.1	3.47	0.812	7.56	16.9
11/10/2003	25.67	41.01	26.22	79.6	5.6	0.601	7.72	11.9

Table 3-1: Water quality data obtained from data sondes at water quality monitoring stations. Temp = Temperature in °C; Sal=salinity; DOsat=oxygen saturation in %; DO= oxygen saturation in mg/l; Depth= depth in meters at time of sampling (an indirect measure of tidal position); pH and Turb= Turbidity.

RESULTS

Adaptation of the Assay to Environmental Samples

Testing of the assay was performed to determine the potential effect of varying salinity on assay results. The MPIA was performed with a Mitomycin C positive control and negative controls with salinities ranging from 0 (freshwater), up to 35 (average seawater salinity). The positive control demonstrated significant induction when compared to the negative controls, regardless of salinity. All of the negative controls were nearly identical, demonstrating no response to varying salinities (data not shown). This insensitivity of the assay to salinity variation was important due to the wide ranges in salinity observed routinely in Reserve water samples.

The initial step in adaptation of the MPIA to environmental samples was the determination of the optimal technique for sample preparation. The goal was to eliminate interference with the assay isolate from ambient bacteria and viruses, while still maintaining the optimum level of sensitivity of the assay. It has previously been observed with environmental samples that microbial contamination would need to be removed by filtration (Houk and DeMarini, 1988; McDaniel et al., 2001).

The first Reserve water column samples were prepared simultaneously using two filtration protocols. The 0.2 μm filtration removes bacteria, but minimal numbers of ambient viruses. The 0.02 μm filtration removed both bacteria and viruses. Although both methods performed similarly in the MPIA, enumeration of VLP's of both sample preparations demonstrated that numerous ambient viruses were allowed to pass through the 0.2 μm filter, while no viruses were observed in the 0.02 μm filtered sample. Because the assay functioned similarly with either method of preparation, the 0.02 μm filtration protocol was selected to prevent any potential interference with VLP enumeration while performing the assay.

In the first sampling there was no significant prophage induction response in the samples from the Fakahatchee Bay or Blackwater River sites. A statistically significant positive induction response was detected from the Rookery Bay water column sample. The DEP lab detected no

contaminants. However, as previously stated, tests conducted on the first sampling included only a limited range of pesticides plus heavy metals.

Some of the sediment pore water samples in the first sediment field test demonstrated a higher response by the MPIA than the corresponding water column samples. This is not surprising since the sediments are generally considered a repository for contaminants. It should also be mentioned here that the sediment samples throughout the reserve were fine-grained, rich in organic matter, and generally anaerobic. Anaerobic sediments were easily detected by the odor of hydrogen sulfide (Madigan, Martinko, and Parker, 1997).

In this first sediment trial the Rookery Bay and Blackwater River sediment samples were positive for prophage induction at the 90% confidence level. The overlying water column samples were negative. During this sampling, the analytical lab detected arsenic in the sediment samples from the Blackwater River and the Fakahatchee Bay sites. There was an induction response at the Blackwater River site, one of the sites with detected arsenic. However, under reducing conditions like these anaerobic sediments, arsenic would have been in the reduced form, arsine, which is non-toxic (Madigan, Martinko, and Parker, 1997). This is supported by the observation that there was no response to a similar level of arsenic at the Rookery Bay site.

Laboratory testing verified that the MPIA isolate is highly sensitive to the oxidized form of arsenic (Fig. 3-3). The response of the assay organism to $1\mu\text{g ml}^{-1}$ of arsenate (KH_2AsO_4) was much higher than to the positive control Mitomycin C. It was also observed that the level of induction decreased with increasing concentration of arsenic due to increasing toxicity. The MPIA test organism was also observed to be non-turbid (killed) in the well of the microtiter plate, at these higher concentrations. A similar phenomenon is also observed with higher concentrations of Mitomycin C. The fact that the response to the sediment samples containing arsenic was mild to negative supports the contention that the arsenic in these samples was in its reduced form.

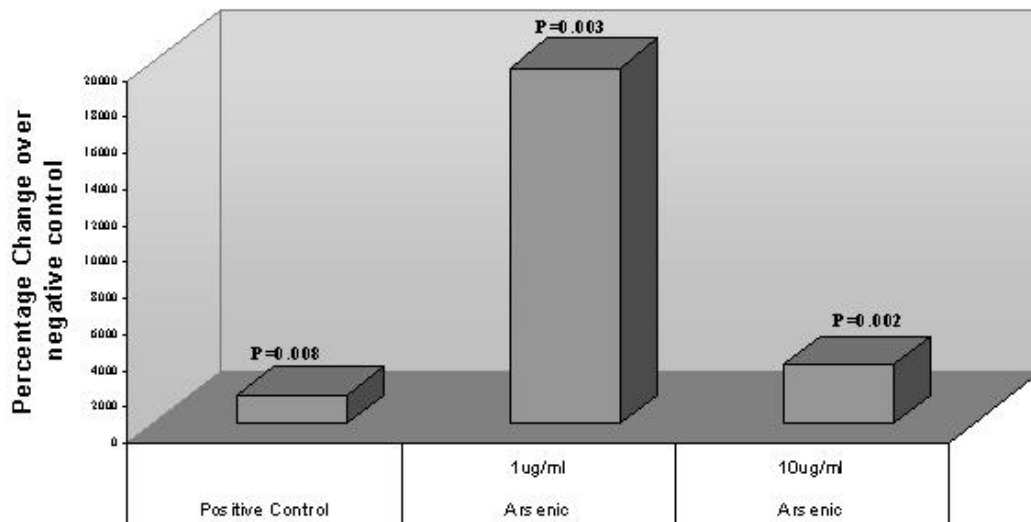


Figure 3-3: The response of the MPIA to arsenate. Note that the response is much higher than the positive control Mitomycin C. At higher doses of arsenic the induction response decreases due to toxicity.

Environmental Positive Control Sampling

Positive control testing was performed after the protocol was adapted for environmental samples. The sites for sampling were selected due to known high contamination, which was based on an extensive survey of Tampa Bay area watersheds (Rose, Paul, and McLaughlin, 1999-2000). The two sites selected were designated as Bullfrog Creek and Sweetwater River (Fig. 3-2). Water column and sediment samples were collected from both sites and tested with the MPIA. Samples for toxicological testing were obtained from the same water and sediments.

At the positive control site excellent correspondence was observed between positive MPIA results and laboratory detection of contaminants (Table 3-2). Several highly toxic compounds were detected at both sites, including some polycyclic aromatic hydrocarbons (PAH's), which are known to result in a positive MPIA response (McDaniel et al., 2001).

Environmental Positive Control Sample:				
Date	Sample Site	Sample Type	MPIA P value	Chemical Analyses
9/17/2003	Bullfrog Creek	Water	NS	ND
		Sediment	0.037	Arsenic, Chlordane
	Sweetwater Creek	Water	0.009	Atrazine
		Sediment	0.058	Benzoanthracene, Benzopyrene, Benzofluoranthene, Benzoperylene, Chrysene, Chlordane

Table 3-2: Comparison of MPIA results and laboratory chemical analysis from a known contaminated site.

Comparison Between MPIA Results and Toxicological Analyses

Environmental testing at the three water quality sites with corresponding laboratory analysis was performed in the reserve over a two-year period. The MPIA and the DEP laboratory testing were performed on both water column and sediment samples. The results of the two years of analysis are found in Table 3-3.

There was fairly good correspondence between positive MPIA results and detection of potentially toxic compounds by laboratory analysis, especially in heavily contaminated samples. Nine positive MPIA results have occurred concurrently with laboratory detection of contaminants in reserve and positive control samples. Nine positive induction responses have occurred without concurrent toxic contaminant detection. This is likely due to the limited range of laboratory testing that it is possible to perform, although false positive MPIA results cannot be ruled out.

Seven detections of a compound by the analytical lab were not accompanied by a positive induction response. However, four of these non-detections were due to arsenic in sediment samples, which is non-toxic under reducing conditions, as noted earlier. Two additional laboratory detections that were not positive by the MPIA were both phthalate compounds, which are common plasticizing agents widely used in many industries and are commonly observed environmental contaminants (Agency, 1987). These compounds are known to be irritants but are not mutagens, they can also be isolated from naturally occurring fungi (Windholz et al., 1976). The only occasion a potentially bioactive toxin was not detected by the MPIA was one sample containing hexazinone (Table 3-3, 10/13/03 sampling).

Field Sampling Data, Rookery Bay Reserve:								
#	Date	Sample Site	Sample Type	% change	P value	Analysis Performed	Compounds Detected	Salinity
1	8/5/2002		Positive Control		0.007	Mit. C control	N/A	
		Fakahatchee Bay	Water column	165%	NS	P, M	ND	14
		Blackwater River	Water column	184%	NS	P, M	ND	26
		Rookery Bay	Water column	274%	0.003	P, M	ND	25
2	10/25/2002		Positive Control		0.016	Mit. C control	N/A	
		Fakahatchee Bay	Water column	2944%	0.03	P, M	ND	20
		Blackwater River	Water column	192%	0.007	P, M	ND	26
		Rookery Bay	Water column	207%	NS	P, M	ND	32
3	12/19/2002		Positive Control		NS		no metals, other samples lost	
4	1/23/2003		Positive Control		0.001	Mit. C control	N/A	
		Fakahatchee Bay	Water column	N/A	NS	P, M, Org, Herb	ND	28
		Blackwater River	Water column	109%	0.017	P, M, Org, Herb	ND	35
		Rookery Bay	Water column	117%	0.024	P, M, Org, Herb	ND	35
5	3/20/2003		Positive Control		0.005	Mit. C control	N/A	
		Fakahatchee Bay	Sediment	3%	NS	P, M, Org, Herb	As (3.4mg/kg)	
		Blackwater River	Sediment	998%	NS 0.078	P, M, Org, Herb	As (3.2mg/kg)	
		Rookery Bay	Sediment	132%	NS 0.068	P, M, Org, Herb	ND	
		Fakahatchee Bay	Water Column	-42%	NS	P, M, Org, Herb	ND	32
		Blackwater River	Water Column	-36%	NS	P, M, Org, Herb	ND	35
		Rookery Bay	Water Column	-50%	NS	P, M, Org, Herb	ND	35
6	5/6/2003		Positive Control		0.022	Mit. C control	N/A	
		Fakahatchee Bay	Sediment	-63%	NS	P, M, Org, Herb	ND	
		Blackwater River	Sediment	-76%	NS	P, M, Org, Herb	Bis(2-ethylhexyl)phthalate	
		Rookery Bay	Sediment	-55%	NS	P, M, Org, Herb	ND	
		Fakahatchee Bay	Water Column	-75%	NS	P, M, Org, Herb	ND	32
		Blackwater River	Water Column	-39%	NS	P, M, Org, Herb	ND	35
		Rookery Bay	Water Column	-21%	NS	P, M, Org, Herb	ND	35
7	6/23- 6/24/2003		Positive Control		0.034	Mit. C control	N/A	
		Fakahatchee Bay	Sediment	-50%	NS	P, M, Org, Herb	ND	
		Blackwater River	Sediment	130%	NS	P, M, Org, Herb	ND	
		Rookery Bay	Sediment	139%	NS	P, M, Org, Herb	Diethyl phthalate, Sr (694mg)	
		Fakahatchee Bay	Water Column	-17%	NS	P, M, Org, Herb	ND	17.5
		Blackwater River	Water Column	159%	NS	P, M, Org, Herb	ND	25
		Rookery Bay	Water Column	-6%	NS	P, M, Org, Herb	ND	26
8	7/22/2003		Positive Control		0.001	Mit. C control	N/A	
		Rookery Bay	Sediment	11%	NS	P, M, Org, Herb	As (6.7mg/kg)	
		Rookery Bay	Water	65%	NS	P, M, Org, Herb	ND	24
8	8/11/2003		Positive Control		0.007	Mit. C control	N/A	
		Fakahatchee Bay	Sediment	230%	0.024	P, M, Org, Herb	As (4.4mg/kg)	
		Blackwater River	Sediment	5%	NS	P, M, Org, Herb	As (3.1mg/kg)	
		Fakahatchee Bay	Water Column	148%	0.049	P, M, Org, Herb	ND	2
		Blackwater River	Water Column	297%	0.024	P, M, Org, Herb	Hexazinone (0.21)	5
9	10/13/2003		Positive Control		0.0001	Mit. C control	N/A	
		Fakahatchee Bay	Sediment	1894%	0.027	P, M, Org, Herb	As (3.9mg/kg)	
		Blackwater River	Sediment	-0.40%	NS	P, M, Org, Herb	As (6.5mg/kg)	
		Rookery Bay	Sediment	2920%	0.001	P, M, Org, Herb	As (4.1mg/kg)	
		Fakahatchee Bay	Water Column	68%	NS	P, M, Org, Herb	ND	5
		Blackwater River	Water Column	130%	NS	P, M, Org, Herb	ND	6
		Rookery Bay	Water Column	237%	NS	P, M, Org, Herb	Hexazinone (0.18)	15
10	11/10/2003		Positive Control		0.001	Mit. C control	N/A	
		Fakahatchee Bay	Sediment	60%	NS	P, M, Org, Herb	ND	
		Blackwater River	Sediment	82%	0.013	P, M, Org, Herb	ND	
		Rookery Bay	Sediment	678%	0.018	P, M, Org, Herb	ND	
		Fakahatchee Bay	Water Column	104%	0.035	P, M, Org, Herb	ND	4
		Blackwater River	Water Column	1851%	0.004	P, M, Org, Herb	Hexazinone (0.16)	5
		Rookery Bay	Water Column	119%	0.028	P, M, Org, Herb	ND	26

Table 3-3: MPIA and analytical laboratory testing results. A MPIA response is considered positive when $P < 0.05$. Positive environmental assay responses are marked in bold face type. P indicates testing for organonitrogen and organochlorine pesticides, M=heavy metals; Org=priority organic compounds (including PAH's and PCB's); Herb=herbicides.

One potentially contaminated Naples Bay site outside the reserve boundary was evaluated using the MPIA alone. This site was located at a water quality station on a busy waterway in Naples Bay adjacent to Naples City Dock (Naples Bay water quality station, Fig. 3-1). The area is characterized by heavy boat traffic and potential contamination from fuels, oils, and boat toilet discharges.

At the Naples Bay site, all of the water column samples demonstrated a statistically significant positive MPIA response with an average percentage change of 227%. Two of the three sediment samples were also positive with an average percentage change of 244%. These results suggest that it is likely some type of bioactive contaminant is present at this site. It was also observed that there was a statistically significant level of variability within this single set of samples ($P < 0.05$). Water column samples were less variable than the sediment samples, which is expected. This indicates that multiple samples should be screened from a single site similarly to the sampling regime utilized for the reserve sites in order to obtain a more consistent picture of the level of contamination.

Laboratory Verification of Positive MPIA Results

In addition to arsenate and PAH's, to which the assay has a known sensitivity, the MPIA demonstrated positive results in conjunction with detection of Fenthion, Endosulfan, Atrazine, Chrysene, Chlordane and Hexazinone. Laboratory testing has verified that the MPIA is sensitive to Fenthion, Endosulfan, Atrazine and Chrysene at levels similar to those detected by the DEP lab (data not shown). Testing with Chlordane and Hexazinone are pending.

DISCUSSION

In general, the reserve was relatively free of mutagenic contaminants. The most common observed pollutants were the pesticide hexazinone in water column samples and arsenic in sediment samples. Toxicological testing in the Reserve did not reveal any contamination by herbicides, and minimal contamination by pesticides. In addition, there were no

incidents of detected PAH's or PCB's, which were major compounds of concern to reserve managers. The analysis determined a relatively low level of pollution throughout the Reserve, however some contamination was observed at the supposedly pristine Fakahatchee Bay reference site.

All MPIA samplings were compared to water quality data obtained at the same times and sampling sites. Multivariate analysis of the MPIA assay parameters (including VLP's ml-1, percentage change and whether the testing was positive or negative) was compared to all water quality parameters to determine if the general water quality condition had any effect on the assay. All water quality parameters for the three reserve sampling sites are found in Table 1. No correlation was observed between the data sets, indicating that the general water quality did not interfere with assay results.

In conclusion, the MPIA appears to be a good screening tool for detection of bioactive contamination in marine water column and sediment samples. The assay appeared to perform particularly well with heavily contaminated samples. Over a two-year period of reserve sampling, the assay demonstrated a positive prophage induction response in conjunction with toxic compounds that were also detected by toxicological testing. The assay failed to show a prophage induction response to samples with detected compounds that were not highly toxic or mutagenic. In addition, no interference was observed due to varying salinity or general water quality parameters. Due to the low cost of the assay, in comparison to toxicological testing, the MPIA is a useful screening tool for determining areas that may contain potential contamination and target them for more detailed examination.

CHAPTER 4

Lysogeny in Marine *Synechococcus*

The following chapter has been peer reviewed and published essentially in this form in the journal *Nature* (Volume 415, p. 496)

CHAPTER SUMMARY

Lysogeny is an alternative to lytic viral infection in bacteria and occurs when a viral genome is stably maintained as a prophage within its host (Ackermann and DuBow, 1987). Here we report the occurrence of lysogeny in natural populations of autotrophic picoplankton and show a seasonal pattern to this phenomenon. Because lysogeny confers immunity to infection by related viruses (Ackermann, 1987), we suggest that this process accounts for the resistance to viral infection often observed in common forms of autotrophic picoplankton (*Synechococcus*) (Waterbury and Valois, 1993).

MATERIALS AND METHODS

The viral reduction technique of Weinbauer and Suttle (Weinbauer and Suttle, 1996) was employed to reduce levels of indigenous extracellular cyanophage. Environmental samples were pre-filtered through a 0.2 μm filter in a sterile filtration apparatus to remove a majority of the free viruses. The retentate was then returned to its original volume by addition of virus-free seawater prepared concurrently by 0.02 μm filtration of the same sample. The samples were divided and the inducing agent (Mitomycin C) was added to the treatment samples.

To discriminate the cyanophage from the total population of all types of environmental viruses, we used a microtiter dilution technique (Suttle and Chan, 1994). The samples were first 0.2 μm filtered to remove any zooplankton grazers. A dilution series was prepared followed by the addition of the susceptible host organism (*Synechococcus* WH7803) in the exponential-phase of growth. After a week to ten days incubation all the dilutions were scored as positive or negative for host lysis and the viral titer was determined by the use of a most probable number (MPN) program (Hurley and Roscoe, 1983). The presence of viral particles in the lysed wells was verified by SYBR Gold staining followed by epifluorescence microscopy (Noble and Fuhrman, 1998). Phosphate enriched samples were prepared by the addition of 10 $\mu\text{mol/liter}$ inorganic phosphate (NaH_2PO_4) to viral reduced samples followed by incubation in either ambient daylight or in the dark.

RESULTS

A seasonal study of prophage induction in cyanobacteria was performed to detect the presence of lysogeny in natural *Synechococcus* populations and the response of this process to changing environmental conditions in Tampa Bay, Florida from November 1999 to October 2000. Cyanophage abundance was determined by microtiter plate dilution series with addition of a host organism, *Synechococcus* st. CCMP 1334 (WH7803, DC2), followed by use of a most probable number (MPN) program. SYBR Gold staining followed by epifluorescence microscopy verified the presence of viral particles in lysed wells. To determine the presence of lysogens, water samples

were assayed for prophage induction using Mitomycin C as the inducing agent. The viral reduction technique of Weinbauer and Suttle (Weinbauer and Suttle, 1996) was employed in preparation of samples to increase the sensitivity of the assay.

A statistically significant level of prophage induction in natural populations of *Synechococcus* was revealed on six separate occasions in response to Mitomycin C exposure (Fig. 4-1). Prophage induction, measured as percentage change over control cyanophage level, was inversely correlated with cyanobacterial abundance and primary productivity ($r = -0.502$, $P=0.0123$ and $r = -0.4109$, $P = 0.0461$, respectively. Correlations were determined by multiple regression analysis of arcsine transformed percentage data and all other measured parameters). Induction events occurred primarily during the late winter months, during times of decreased host abundance (Fig. 4-1). One induction event was also observed in late August, which preceded a secondary fall "bloom" in *Synechococcus* abundance. This event demonstrated that the presence of prophage induction in *Synechococcus* was not merely an artifact due to lower cyanophage abundances during winter months.

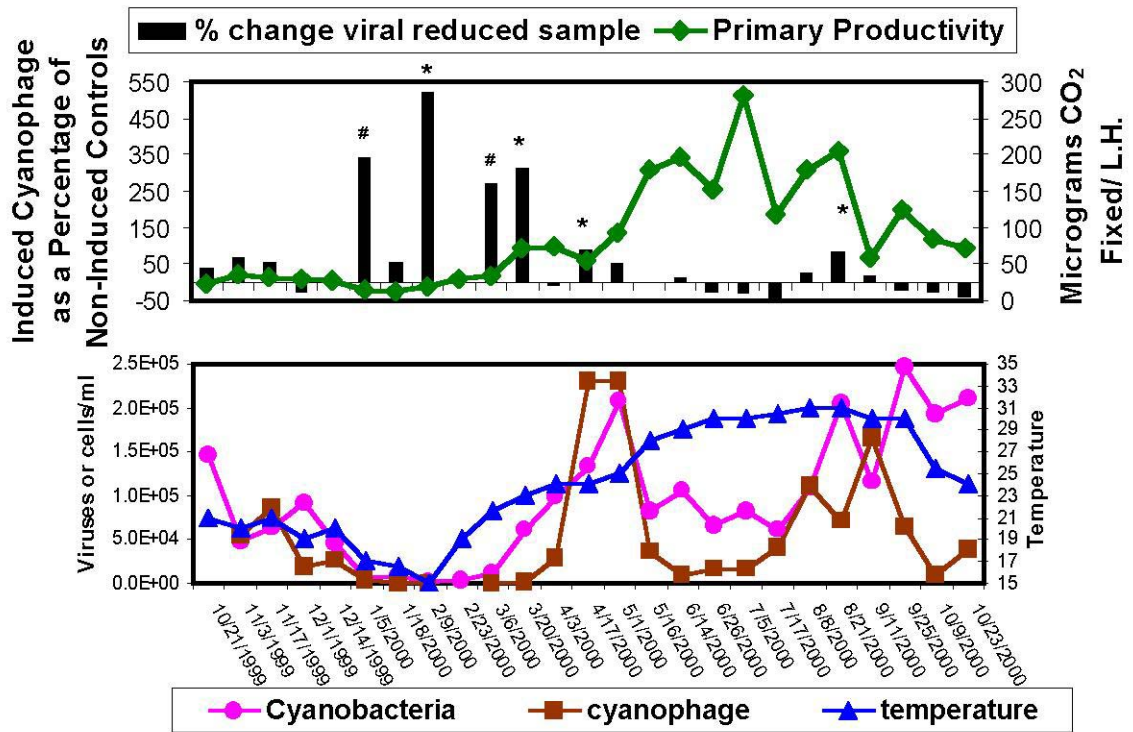


Figure 4-1: Prophage induction of *Synechococcus* populations (closed bar) compared to *Synechococcus* abundance (●), cyanophage counts (■), primary productivity (◆), and temperature (▲). Results are expressed as percentage change in treatment compared to control (* and # indicate statistical significance by paired t-test at the 95% and 90% confidence interval, respectively).

A seasonal pattern is consistent with occurrence of lysogeny at times of low host abundance, periods of resource limitation or adverse environmental conditions to ensure viral survival (Ackermann and DuBow, 1987). Another reason for the limited occurrence of lysogeny in the summer months is the possibility that greater UV exposure or higher temperatures already caused induction of many of the prophage.

The MPN method of cyanophage detection has been demonstrated to have a similar level of precision to other methods of viral enumeration (Cottrell and Suttle, 1991). However, this method will only detect a subset of the lysogenic *Synechococcus* population (i.e. phage infective for *Synechococcus* st. CCMP 1334) and the total number of inducible cyanophages is certainly higher. Thus, our values are a conservative estimate of the actual number of lysogenic *Synechococcus* present.

Viruses are very abundant in the marine environment (Bergh et al., 1989), infecting both heterotrophic and autotrophic microbial populations (Suttle, 1992), modulating microbial production and in some cases causing algal bloom termination (Suttle and Chan, 1993). Previous studies of cyanobacteria have revealed that they can be resistant to lytic infection by co-occurring cyanophage (Waterbury and Valois, 1993). *Synechococcus* strains have also been demonstrated to be less sensitive than larger species of phytoplankton to the photosynthetic inhibition produced by the viral fraction of seawater (Suttle, 1992). We hypothesize that a plausible explanation for these observations may be homoimmunity conferred by lysogeny.

CHAPTER 5

Effect of Nutrient Addition and Environmental Factors on Prophage Induction in Natural Populations of Marine *Synechococcus*.

The following chapter has been peer reviewed and published essentially in this form in the journal *Applied and Environmental Microbiology* (2005, Volume 71, p. 842-850)

CHAPTER SUMMARY

A series of experiments were conducted with samples collected in both Tampa Bay and the Gulf of Mexico to assess the impact of nutrient addition on cyanophage induction in natural populations of *Synechococcus*. The samples were viral-reduced to decrease the background level of cyanophage and then either left untreated or amended with nitrate, ammonium, urea, or phosphate. Replicate samples were treated with Mitomycin C to stimulate cyanophage induction.

In five of the nine total experiments performed, cyanophage induction was present in the non-nutrient amended control samples. Stimulation of cyanophage induction in response to nutrient addition (phosphate) occurred in only one Tampa Bay sample. Nutrient additions caused a decrease in lytic (or control) phage production in three of three offshore stations, in one of three estuarine experiments, and in a lysogenic marine *Synechococcus* in culture.

These results suggest that the process of cyanophage induction as an assay of *Synechococcus* lysogeny was not inorganic nutrient-limited, at least in the samples examined. More importantly, it was observed that the level of cyanophage induction (cyanophage ml⁻¹) was inversely correlated to *Synechococcus* and cyanophage abundance. Thus, the intensity of the prophage induction response is defined by ambient population size and cyanophage abundance. This corroborates prior observations that lysogeny in *Synechococcus* is favored during times of low host abundance.

INTRODUCTION

Bacteriophages are excellent survivors having evolved different strategies for coping with environmental challenges. In addition to the familiar lytic infection, certain temperate phages can alternatively become integrated into the host chromosome as a prophage. Lysogeny has been extensively studied in heterotrophic bacteria. The most well known example is the *Escherichia coli*/ λ system, which has been studied in detail for over fifty years, accounting for much of what is known today about the molecular basis of the interactions between temperate phage and their host.

An extensive survey of cultured, heterotrophic bacteria estimated that approximately 50% of bacterial strains were lysogenic (Ackermann, 1987). Recent sequencing of bacterial genomes has also conclusively demonstrated that integrated viral genomes are common. Fifty-one of 82 genomes examined were determined to carry prophages, and within those 51 genomes, a total of 230 recognizable putative prophages were identified (Casjens, 2003).

Initially, lysogeny was believed to be of limited importance in marine environments [Reviewed in (Mann, 2003)]. However, it has been demonstrated experimentally that a large number of heterotrophic marine bacterial strains contain inducible prophage (Jiang and Paul, 1996b; Mann, 2003). Furthermore, studies have demonstrated temporal variations in the prevalence of lysogeny in heterotrophic bacterial populations (Cochran and Paul, 1988; Williamson et al., 2002).

Lysogeny has been described as an adaptation of viruses allowing for survival during adverse conditions, especially when there is low host abundance. Mathematical modeling has demonstrated that there is a reciprocal relationship between bacterial diversity and viral abundance, with lysogeny theorized to act as the repository preventing viruses from disappearing altogether during periods of low host abundance (Thingstad, 2000).

In addition to heterotrophic bacterioplankton, the marine environment contains many autotrophic prokaryotes. Since cyanobacterial picoplankton were first described in 1977 it has become increasingly evident that they play an essential ecological role in the marine environment. These picoplankton are distributed throughout the oceans except the polar

Regions, and are most abundant in tropical and temperate waters. It has been estimated that the *Synechococcus* component of the picoplankton may account for up to 25% of worldwide primary productivity and up to 60% of the total primary productivity at the 1% light level (Waterbury et al., 1986).

Despite the obvious ecological importance of picoplankton, there has been very little research to date on lysogeny in these autotrophic organisms. Much of the investigation to date on cyanophage and cyanobacteria has focused on lytic interactions and characterization of cyanophage. Understanding lytic interactions between cyanophage and cyanobacteria is important because viral infection has been found to be a significant factor in the mortality of cyanobacteria. Viral lysis is responsible for a large but variable fraction of mortality in cyanobacteria, estimated to be on the order of 30% (Proctor and Fuhrman, 1990). Examination of natural cyanobacterial samples has shown that up to 5% of cyanobacteria contain mature phage (Proctor and Fuhrman, 1990).

Viruses have also demonstrated the potential to decrease primary productivity in phytoplankton populations. The addition of seawater concentrates of the 0.002-0.2 μm size fraction to phytoplankton cultures resulted in an average decline in primary productivity of 44% (Suttle, Chan, and Cottrell, 1990). In these experiments *Synechococcus* species were much less sensitive than other types of phytoplankton to the inhibitory effect of viral concentrates (Suttle, 1992). It is possible that this increased resistance to phage pressure is due to the presence of homoimmunity resulting from lysogeny in the cyanobacterial community. The occurrence of lysogeny in this ubiquitous phytoplankton may have important consequences in carbon fixation and microbial loop processes.

Early research verified lysogeny in the freshwater cyanobacterium *Plectonema boryanum* (Rimon and Oppenheim, 1975). These cyanobacterial isolates demonstrated spontaneous induction of prophage as well as induction when treated with Mitomycin C or glutathione (Cannon, 1971). Lysogeny has also been reported in the marine filamentous form *Phormidium* (Ohki and Fujita, 1996). Some preliminary evidence also suggests that lysogeny in cyanobacteria may have

a similar molecular basis to that of heterotrophic organisms (Owtrim and Coleman, 1987a). At this time, it is unknown how widespread lysogeny may be in autotrophic prokaryotes.

Cyanophages that infect marine *Synechococcus* strains have been confirmed to have a very high genetic diversity [reviewed in (Mann, 2003a)]. In addition, this overall species richness was observed to be higher in the winter and lowest in the summer, despite higher overall abundance in the summer. This seasonal variation in richness of genetic types may be partially due to presence or absence of naturally occurring prophage induction in *Synechococcus*.

Lysogeny has been recently documented in natural populations of marine *Synechococcus* (McDaniel et al., 2002; Ortmann, Lawrence, and Suttle, 2002a). A seasonal pattern was observed in the occurrence of lysogeny in *Synechococcus*, with a prevalence of positive inductions in late winter to early spring (McDaniel et al., 2002).

In addition to prophage induction in natural populations, induction of cyanophage has been demonstrated from a cultured marine *Synechococcus* species isolated from the coastal waters off Kyushu, Japan. Cultures of this cyanobacterial isolate produced prophage when induced by UV light, Mitomycin C (Sode et al., 1994b), and a heavy metal (Sode, Oonari, and Oozeki, 1997a). Okhi (1999) reported Mitomycin C stimulated prophage induction in cultured and natural samples of the filamentous cyanobacterium *Trichodesmium* (Okhi, 1999).

The factors controlling the switch from lysogenic to lytic existence in cyanophage are currently unknown. One hypothesis suggests that lysogeny is a survival mechanism activated in response to resource limitation. Phosphate limitation is one potential control over both lytic and lysogenic viral replication. For example, viral replication was inhibited under phosphate limited conditions in the marine coccolithophorid phytoplankter *Emiliana huxleyi* (Bratbak, Egge, and Heldal, 1993).

Besides being a factor possibly limiting lytic viral replication, phosphate amendment has been shown to enable prophage induction on occasion in natural populations of heterotrophic bacteria (Williamson et al., 2002). In *Synechococcus*, nutrient availability, especially phosphate, affects the kinetics of cyanophage infection and may be responsible for the switch from lysogeny to lytic viral production (Wilson, Carr, and Mann, 1996; Wilson, Turner, and Mann, 1998). It has

also been suggested that the high affinity and uptake rate for inorganic phosphate in *Synechococcus* explains its predominance in the phytoplankton (Ikeya et al., 1997; Moutin et al., 2002b).

A recent study of water column viral interactions *Synechococcus*, and its close relative *Prochlorococcus*, demonstrated decreasing cyanophage titers along a transect from coastal to oligotrophic waters (Sullivan, Waterbury, and Chisholm, 2003a). The researchers in this study suggested that decreasing levels of nutrients favored lysogeny rather than lytic infection.

The hypothesis was that lysogeny in *Synechococcus* might be more prevalent under nutrient limited conditions, and that cyanophage induction could be stimulated by nutrient addition. Experiments were performed in oligotrophic and coastal environments and in culture to determine if nutrient availability affects the switch from lysogeny to lytic viral production.

MATERIALS AND METHODS

Locations and Sampling

The initial group of samples was obtained as a part of a 13-month seasonal study of prophage induction in cyanobacteria during 1999-2000. Natural seawater samples were obtained bi-monthly from the St. Petersburg Pier located on the Tampa Bay estuary (Fig. 5-1). An additional sampling occurred in Tampa Bay in July 2002.

Samples were also obtained from the Gulf of Mexico during a research cruise in July 2001 representing differing nutrient regimes associated with the Mississippi River plume as it entered the oligotrophic Gulf of Mexico (Wawrik and Paul, 2004). Figure 5-1 is a seven-day composite Sea-Viewing Wide Field-of-View Sensor (SeaWiFS) image of the Mississippi River plume marked with the locations of the sampling stations. Cyanophage Induction experiments were performed at stations 1, 3, 7, and 10 with nutrient amendment inductions performed at stations 3, 7 and 10.

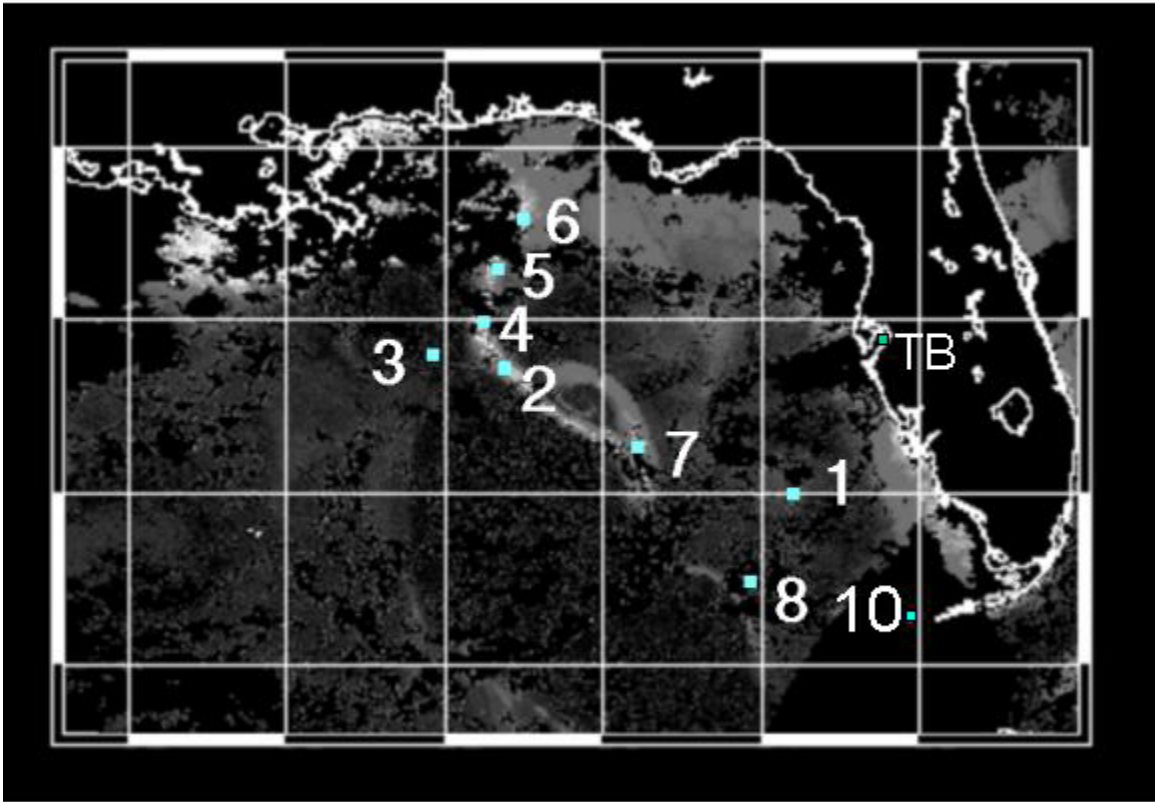


Figure 5-1: SeaWiFS image of the Gulf of Mexico with sampling locations. The coastal plume of the Mississippi River is entrained by the Gulf loop current and carried southward along the Florida shelf.

***Synechococcus* Counts**

Synechococcus counts were performed using epifluorescence microscopy at blue excitation as previously described by Vernet et al (1990) (Vernet, Mitchell, and Holm-Hansen, 1990a).

Cyanophage Counts

To differentiate the cyanophage from the total viral population the Most Probable Number (MPN) method was utilized (Suttle and Chan, 1994a). A one to five dilution series of the environmental or prophage induction treatment sample was prepared using 96-well microtiter plates (Costar, Corning Inc.). A susceptible *Synechococcus* host was then freshly diluted 1:10 and placed in each well (either *Synechococcus* isolate WH7803, our own isolate GM9901, or both). Control plates were prepared similarly using sterile SN media in the first column of wells. Three replicate treatment and control plates were prepared from each site. The plates were incubated until dense growth of the host organism was evident (10-14 days). Wells were scored as positive for virus if lysis of the host organism was evident. Viral abundance was calculated for each plate using an MPN program (Hurley and Roscoe, 1983).

Prior to being used in the MPN assay, both *Synechococcus* isolates were tested with a dilution series of Mitomycin C. Slight growth inhibition was observed at the highest experimental concentrations, but not at the diluted levels used in the assay. No prophage/cyanophage induction was observed in either isolate (data not shown).

Prophage induction

The samples for prophage induction were pretreated by the technique of viral reduction (Weinbauer and Suttle, 1996b). Briefly, each sample was filtered through a 0.2 μm filter to a volume of approximately 5 ml to remove most of the ambient viruses. Virus-free (0.02 μm -

filtered) water prepared from the same sample was added and the volume reduced a second time. The retentate was then returned to its original volume by addition of virus-free seawater. The reconstituted sample was then divided into aliquots and incubated with and without nutrient amendments. The nutrient amendments consisted of a 50 μM final concentration of ammonium (NH_4Cl), nitrate (NaNO_3) or urea, or a 10 μM final concentration of phosphate (KH_2PO_4). All chemicals used were obtained from Sigma Chemical Co., St. Louis, MO. The compounds were prepared using reagent-grade water and sterilized by autoclaving before use. Treatment samples were also amended with the inducing agent Mitomycin C at a concentration of 1 $\mu\text{g ml}^{-1}$ both with and without the same nutrients as the control samples.

The final group of environmental samples was obtained from Tampa Bay. Although *Prochlorococcus* and *Synechococcus* have been demonstrated to be poor food items for common types of zooplankton (Christaki, 2002), these samples were pre-filtered (1 μm) to remove potential interference from grazing organisms.

Viral Production

Cyanophage viral production was calculated by the dilution technique as previously described (Wilhelm, Brigden, and Suttle, 2002). Measurements were based on the MPN cyanophage numbers for both non-nutrient amended and nutrient amended samples.

Nutrient Analysis

Ambient nutrient concentrations were measured at stations 1, 3, and 7 for both filtered and unfiltered water samples. All water samples were stored in 30 ml polycarbonate bottles at -20° C until analyzed. The analytical laboratory of the Virginia Institute of Technology performed the nutrient analyses.

Statistical Analysis

Two types of statistical analysis were performed. Firstly, treatment and control cyanophage and *Synechococcus* counts were evaluated by paired t-test between samples using Minitab statistical software. Induction results and environmental parameters were compared using linear regression and Chi Square analysis, also using Minitab. Secondly, multivariate analysis of cyanophage induction parameters and measured environmental parameters were performed using Primer v.5.2.9 software (Primer-E Ltd., Plymouth Marine Laboratory, U.K. www.primer-e.com). Initially, the similarity matrices of both the cyanophage induction parameters and environmental parameters, constructed utilizing normalized Euclidian distances, were compared to determine if a statistically significant relationship existed between the matrices utilizing the RELATE test (sample statistic ρ). The matrices were then optimized using both fourth-root and log transformations and compared by the BIOENV test to determine which environmental parameters best accounted for the observed distribution of cyanophage induction.

Prophage Induction in *Synechococcus* Cultures

Preliminary phosphate-enrichment induction experiments were also performed with a known lysogenic *Synechococcus* isolate (strain GM 9914) in our culture collection. The isolate in log-phase growth was divided into four separate flasks and amended in a similar fashion to the protocol described for the cruise experiments. One flask received no amendment and served as control. One flask received $0.5 \mu\text{g ml}^{-1}$ Mitomycin C, one received a $10 \mu\text{M}$ phosphate amendment (KH_2PO_4), and the fourth flask received both phosphate and Mitomycin C. Viral abundance for each treatment was monitored by SYBR-Gold staining (Noble and Fuhrman, 1998).

RESULTS

To determine if elevated phosphate levels could stimulate or enable prophage induction in *Synechococcus*, phosphate enriched treatments were added to the latter part of a thirteen-month seasonal study on lysogeny in *Synechococcus*. Of the nine phosphate-enrichment experiments performed, cyanophage induction was observed in only three (Fig. 5-2).

In the September sampling, the phosphate enrichments appeared to enable cyanophage induction. This effect was observed in the dark incubation with phosphate amendment in combination with the inducing agent Mitomycin C and not with the phosphate enrichment alone, or Mitomycin C alone. In the July sampling, prophage induction was only observed in the phosphate-enriched, Mitomycin C sample. However, the increase in the level of cyanophage induction observed, measured as percentage change over control, was attributable to a decrease in the phosphate-enriched controls. This decrease was not statistically significant in these initial studies and was also not observed in the September positive induction. In addition, the level of cyanophage in both the nutrient-enriched and non-enriched Mitomycin C samples were similar. In the August positive induction experiment, prophage induction occurred at a similar level in response to Mitomycin C both in the presence and absence of phosphate amendment, indicating that phosphate did not stimulate or enable prophage induction.

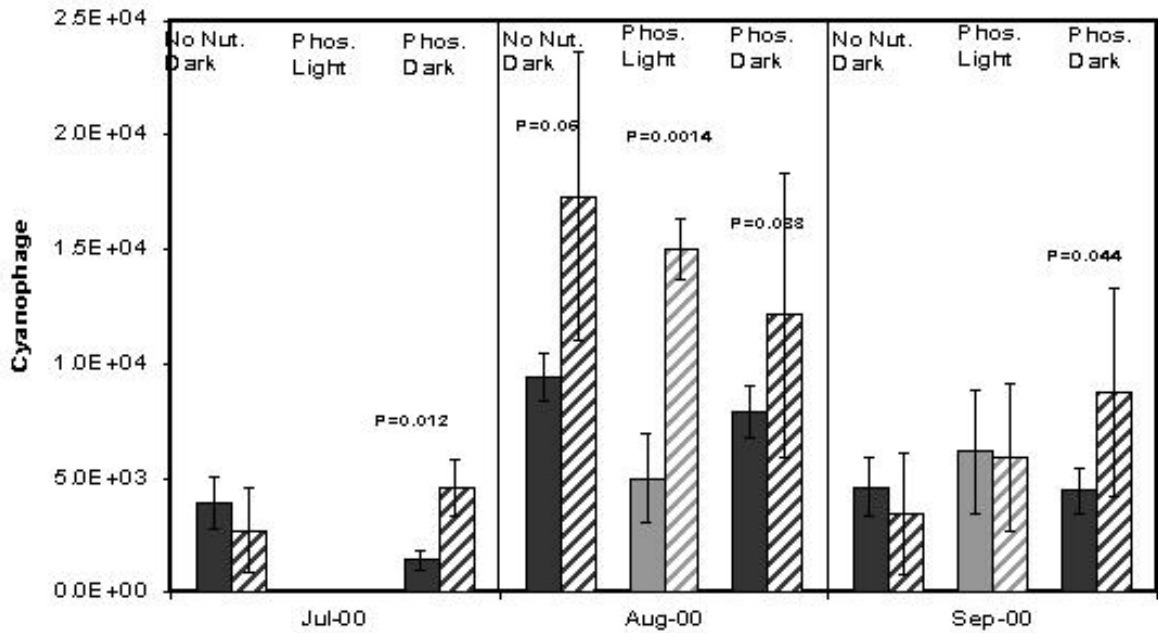


Figure 5-2: The effect of phosphate enrichment on prophage induction during the latter part of a seasonal study in Tampa Bay in 2000. Solid bars indicate control samples; crosshatched bars indicate Mitomycin C treatment. Experimental conditions included non-nutrient amended dark incubations (No Nut. Dark), and phosphate-amended samples incubated both in the light (Phos. Light), and in the dark (Phos. Dark). Numbers above the bars indicate level of statistical significance.

To further investigate the effect of nutrients on lysogeny, cyanophage induction experiments were conducted during a research cruise along a transect of the Mississippi River plume (Fig. 5-1, map) during the summer of 2001. A variety of nutrient conditions were available for testing the hypothesis that nutrient status would affect the presence of lysogeny in *Synechococcus* populations and the response to nutrient stimulation.

With the exception of station 10, the cyanophage counts were approximately an order of magnitude less than the *Synechococcus* counts in both offshore and coastal environments (Fig. 5-3). At station 10 the cyanophage counts were higher than the *Synechococcus* counts, as well as being higher than the cyanophage counts at all other stations sampled.

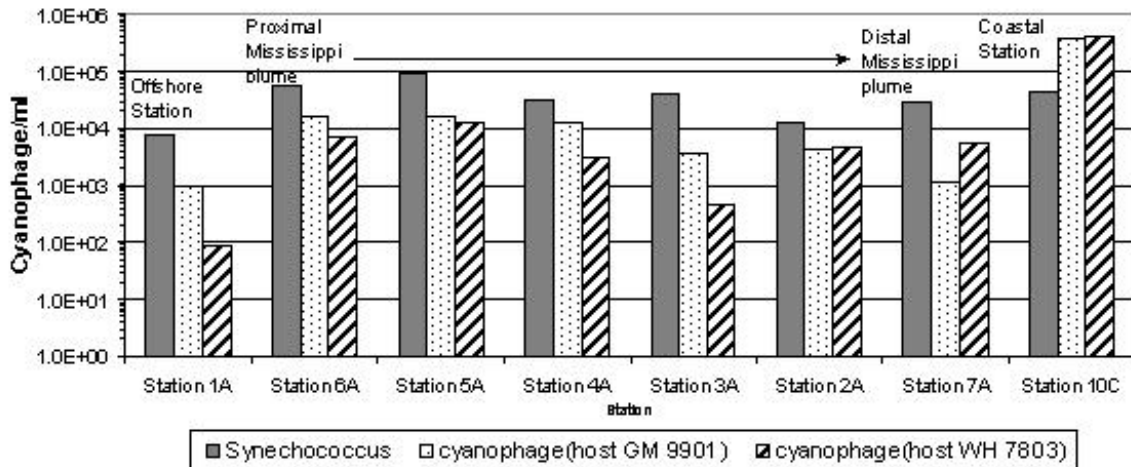


Figure 5-3: Ambient level of *Synechococcus* and cyanophage at all cruise stations sampled in 2001 (see Figure 5-2 for station locations).

Station 1 was an oligotrophic offshore site outside of the Mississippi plume. A large (1463%) increase in cyanophage was observed at this station in response to Mitomycin C treatment ($P < 0.0005$, data not shown). No nutrient amendments were performed at this station. The inorganic N:P ratio of 1.25 suggested that nitrogen limitation may have occurred (Table 5-1). In addition to having the lowest N:P ratio, this station had the lowest overall abundance of *Synechococcus* and cyanophage.

Table 1: Station Characteristics				
Station	Ammonium	Nitrate	Phosphate	N:P
1A	0.32	0.05	0.30	1.25
3A	0.18	2.27	0.27	8.94
7A	0.21	0.34	0.28	1.99

Table 5-1: Station Parameters from Gulf of Mexico Cruise. All values are micromolar concentrations.

Station 3 was located along the edge of the proximal plume of the Mississippi River, with an inorganic N:P ratio of 8.94. The increase in cyanophage was not significant in the non-nutrient amended Mitomycin C treated samples over control samples (baseline cyanophage induction). However, significant inductions were observed at the 90% confidence interval in response to nitrate and ammonium enrichments and at a greater than the 95% confidence interval in response to phosphate enrichment (Fig. 5-4). The numbers of cyanophage produced in response to Mitomycin C induction were very similar in both the nutrient-enriched and non-enriched treatment samples. However, decreases in the cyanophage titer in the nutrient-enriched controls resulted in a calculated significant proophage induction in the nitrate and phosphate amended samples.

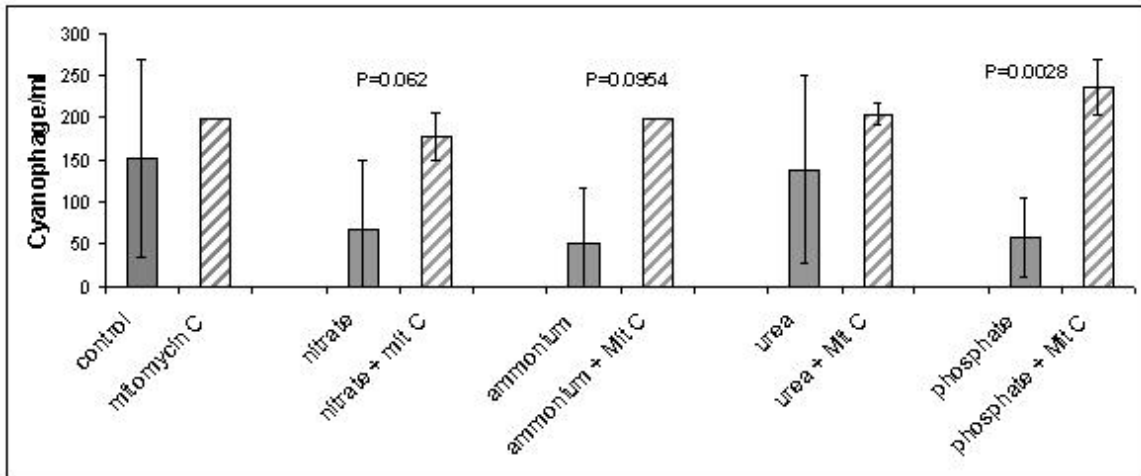


Figure 5-4: Cyanophage induction experiments from cruise station 3. Solid bars indicate control samples; crosshatched bars indicate Mitomycin C treatment. Error bars indicate the standard deviation.

Station 7 was located within the distal Mississippi River plume. The inorganic N:P ratio of 1.99 suggested that nitrogen limitation may have occurred at this station. At this site there was a statistically significant ($P < 0.05$) induction in the baseline non-nutrient amended sample, as well as a statistically significant response to nutrient enrichment. Based on percentage change estimations, the nutrient amended samples demonstrated a higher level of cyanophage induction. Similarly to station 3, this was due to a decrease in the cyanophage titer in all of the nutrient-amended controls (Fig. 5-5). In contrast to station 3, the decrease in the level of cyanophage in the nutrient amended controls in comparison to the non-amended control was statistically significant ($P < 0.05$). No significant inductions were detected at station 10.

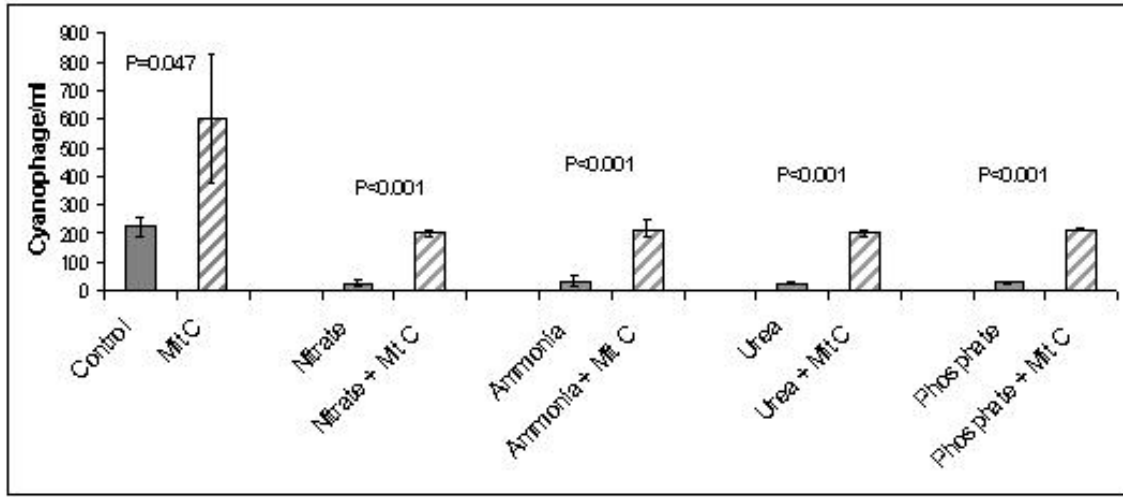


Figure 5-5: Cyanophage induction experiments from cruise station 7. Solid bars indicate control samples; crosshatched bars indicate Mitomycin C treatment. Error bars indicate the standard deviation.

Additional cyanophage induction experiments were performed with Tampa Bay water samples incubated with the nutrient amendments for four hours prior to the addition of Mitomycin C for two reasons. The first reason was to determine the effects of nutrient addition on growth of the host organisms and on viral production rates in nutrient-amended controls (Fig. 5-6). The second reason was to help prevent any potential interaction between the added nutrient and the Mitomycin C.

Growth of the *Synechococcus* was affected by the nutrient amendments (Fig. 5-6). With all three of the nutrient additions, initial growth of the host was slowed in comparison to the control at four hours, with all counts similarly decreased by twenty-four hours. The *Synechococcus* cells in the nutrient-amended samples were also more strongly affected by the Mitomycin C treatment than the non-nutrient amended sample (Fig. 5-6). This decrease in cell abundance in response to Mitomycin C in the nutrient-amended samples at 24 hours was significant at the 94% confidence interval with P values of approximately 0.06 for all three nutrient amendments.

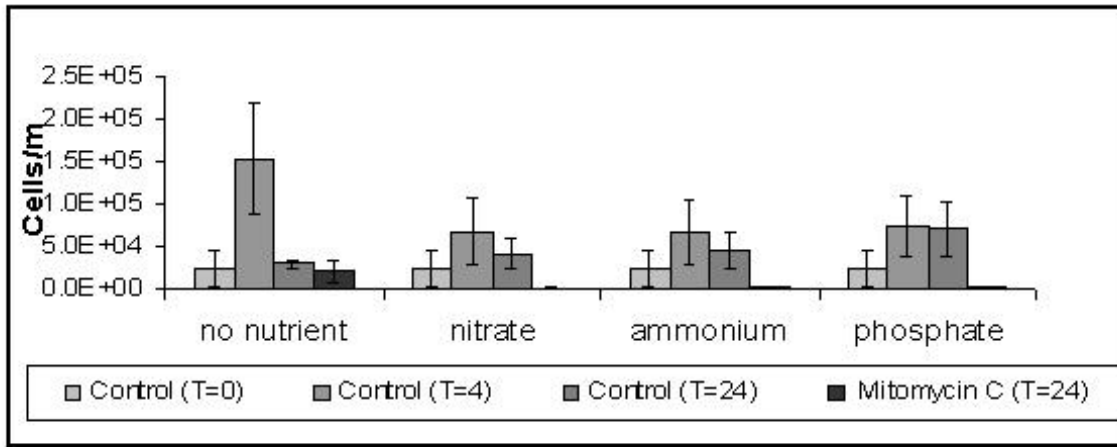


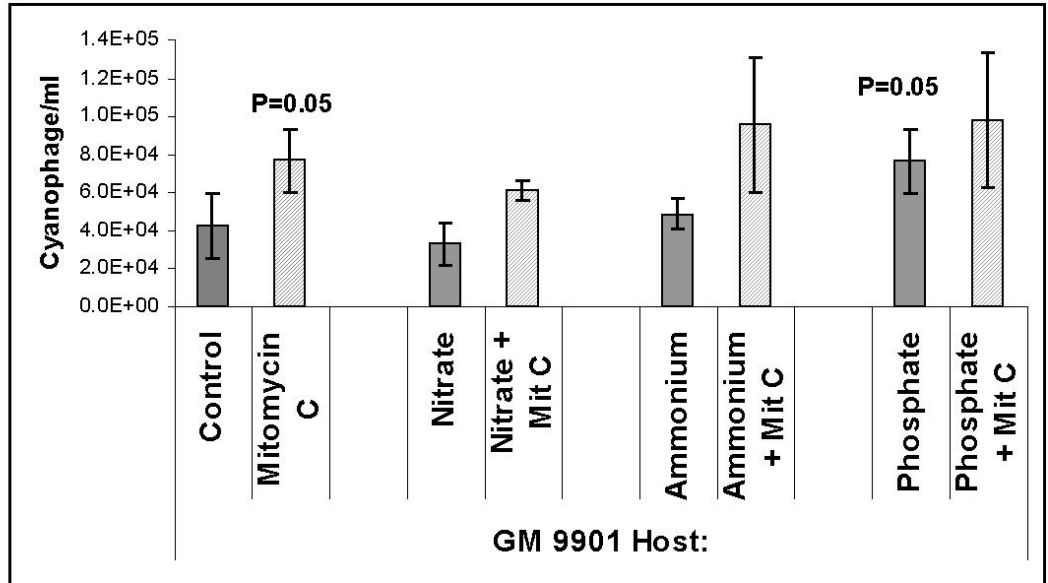
Figure 5-6: *Synechococcus* counts in control and nutrient amended samples from the Tampa Bay induction experiment at four and twenty-four hours, and in Mitomycin C treated samples at 24hours. Error bars indicate the standard deviation.

Four-hour viral production rates were also decreased in the nutrient-amended samples in the additional Tampa Bay experiment. Utilizing host organism GM 9901 to enumerate cyanophage abundance, baseline cyanophage viral production rate was 2.2×10^5 cyanophages hr^{-1} . Addition of nitrate, ammonium, and phosphate decreased the viral production rates to 9.1×10^3 , 9.4×10^3 , and 7.6×10^3 cyanophages hr^{-1} respectively. Utilizing host organism WH 7803 for enumeration, the baseline level of viral production was estimated to be 2.9×10^5 cyanophages hr^{-1} . The same nutrient additions led to the decreased production rates 1.3×10^4 , 3.5×10^3 and 4.6×10^3 cyanophages hr^{-1} , respectively. This decrease in cyanophage production observed with the addition of inorganic nutrients is consistent with the observations of decreased cyanophage abundance in nutrient amended controls in the offshore waters (stations 3 and 7) described above.

In the additional Tampa Bay experiment, a statistically significant level of cyanophage induction ($P < 0.05$) was detected in the non-nutrient amended sample utilizing both *Synechococcus* host organisms (Fig. 5-7A-B). Both host organisms also demonstrated a significant increase in the level of cyanophage in the phosphate-amended non-Mitomycin C sample. Based on our criterion for nutrient stimulated induction, these results indicated that the phosphate amendment might have stimulated lytic cyanophage production in this experiment, but not prophage induction.

The WH 7803 host also enabled detection of a significant increase in cyanophage in the ammonium-amended sample compared to the non-amended control. However, the ammonium plus Mitomycin C sample was significantly higher than the nutrient-amended sample alone. This result indicated that the ammonium enrichment might have stimulated lytic viral production while still enabling detection of Mitomycin C stimulated prophage induction.

A:



B:

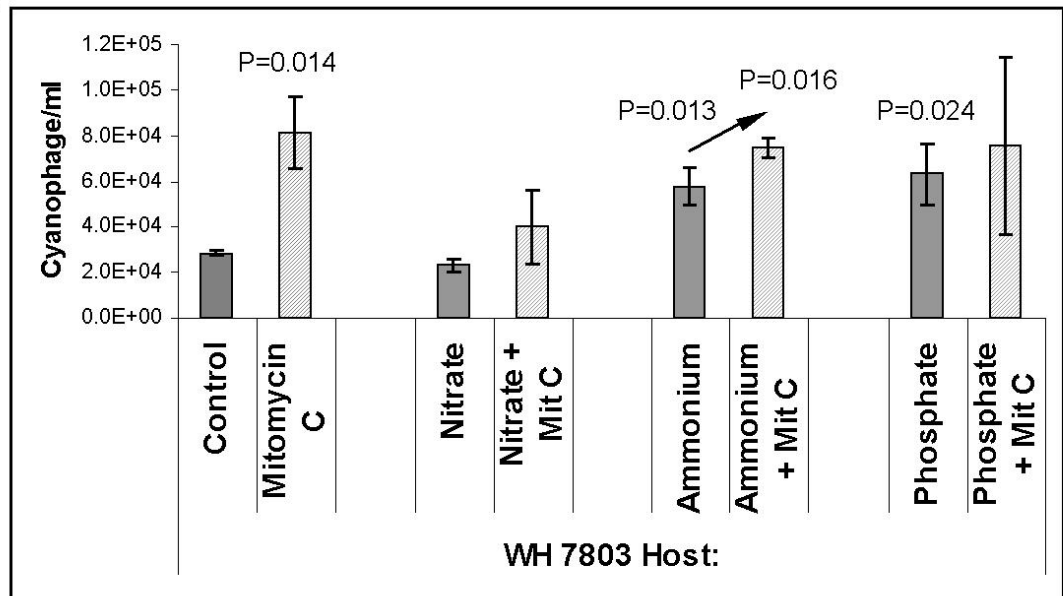


Figure 5-7: Tampa Bay induction experiment with change in the level of cyanophage detected utilizing *Synechococcus* host GM 9901 (5-7A). The experiment was performed concurrently utilizing *Synechococcus* host WH 7803 (5-7B). Solid bars indicate control samples; crosshatched bars indicate Mitomycin C treatment. Numbers above the bars indicate the level of statistical significance with error bars indicating the standard deviation. In the ammonium enriched sample in figure 5-B, the arrow indicates a statistically significant increase (P=0.016) in the sample containing ammonium and Mitomycin C in comparison to the sample amended with Ammonium alone.

In the experiment performed with the lysogenic *Synechococcus* strain GM 9914, a similar decrease in level of viruses was observed in response to the phosphate amendment (Fig. 5-8). A statistically significant prophage induction was observed in response to the Mitomycin C treatment. There was also a significant decrease in the level of virus in the phosphate-amended sample compared to the negative control. In addition, prophage induction appeared to be suppressed in the phosphate plus Mitomycin C-treated sample (Fig. 5-8).

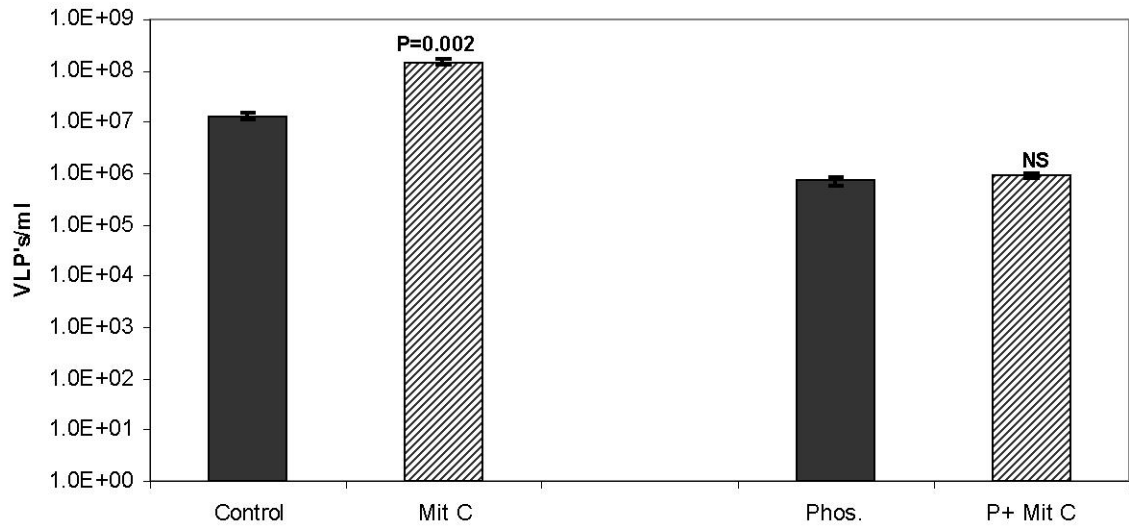


Figure 5-8: Nutrient enrichment, induction experiments with lysogenic *Synechococcus* isolate. Solid bars indicate control samples; crosshatched bars indicate Mitomycin C treated samples. Note the logarithmic scale.

DISCUSSION

Prior work has shown that Mitomycin C prophage induction has occurred variably over space and time in natural populations of both heterotrophic bacteria and picocyanobacteria (McDaniel et al., 2002; Ortmann, Lawrence, and Suttle, 2002a; Williamson et al., 2002). The goal of this study was to determine if the detection of lysogens by this assay might underestimate the occurrence of lysogeny because of nutrient limitation. Cells may lack sufficient nitrogen or phosphorus to enable induction to occur in the presence of this artificial inducing agent. Only one of the Tampa Bay samples (Fig. 5-2) demonstrated greater induction of prophage with nutrient addition (phosphate) plus Mitomycin C rather than Mitomycin C alone.

At the offshore stations 1 and 7, both areas with low N: P ratios, Mitomycin C stimulated prophage induction, without addition of any nutrients. Nitrogen-limited oceanic environments may favor the occurrence of lysogeny. At offshore stations 3, and 7, coastal station 10, Tampa Bay samples and culture samples, nutrient addition resulted in a decrease in viral abundance in non-Mitomycin C treated samples. These results suggest that nutrient addition had a general inhibitory effect on lytic viral production and/or spontaneous induction.

At station 10 there was no detectable cyanophage induction in response to either the nutrients or Mitomycin C. These findings suggest that the cyanophage were already induced by natural conditions at this site leading to the high cyanophage abundances. Alternatively, there may have been no lysogenic *Synechococcus* at this site. There was also a significant decrease in viruses in the nutrient-amended controls at this station.

Unlike the environmental isolates, the phosphate amended plus Mitomycin C treated lysogenic marine *Synechococcus* isolate demonstrated an inhibition of prophage induction. The addition of nutrients and Mitomycin C simultaneously may have led to some synergistic or antagonistic effects between the compounds. However, the different reactions observed in cells in variable physiological condition (i.e., nutrient-limited environmental cells vs. nutrient-replete cultures) argues against this. Further experimentation will be needed to determine this conclusively.

Although Mitomycin C is an artificial way of inducing cyanophage, it does allow the experimental detection of inducible prophage and has been the agent of choice for this purpose for many studies. The criterion for nutrient limitation of the process of cyanophage induction was the observation of a significant increase in samples amended with nutrient plus Mitomycin C over the samples amended with the nutrient alone, in conjunction with no cyanophage induction in samples amended with Mitomycin C alone. Based on our criteria, it appeared that nutrient amendment sometimes inhibited lytic viral production while still allowing Mitomycin C stimulated induction, leading to a relative increase in the level of prophage induction. However, prophage induction stimulated by the nutrient addition alone only occurred once in this study (Tampa Bay seasonal sampling from September).

Nutrient amendments alone may “turn the lysogenic switch” leading to prophage induction with lytic production of cyanophage. This would explain the observation of a significant increase in the level of cyanophage in the nutrient-amended samples in comparison to controls. In this case, if the nutrients alone were stimulating prophage induction, a comparable, significant increase in viruses would be expected in the Mitomycin C treated sample as well, which was not observed. The lack of increase in viruses in the nutrient and Mitomycin C samples was possibly caused by some synergistic effect between phosphate and Mitomycin C. However, this seems unlikely since during the August seasonal experiment and cruise experiments, prophage induction did occur and the nutrient and non-nutrient enriched, Mitomycin C treatments were statistically indistinguishable.

Another comparable set of experiments on heterotrophic bacterial populations in the Gulf of Mexico led to some similar conclusions (Williamson and Paul, 2004). Although the ambient heterotrophic and autotrophic bacterial populations responded differently to addition of nutrient supplements, increases in lytic viral production were observed rather than stimulation of prophage induction. Only one experiment using a 0.1 μ M concentration of phosphate demonstrated clear stimulation of prophage induction. This led to the parallel conclusion that, for the heterotrophic bacterial populations examined, the process of prophage induction is rarely nutrient limited.

Relative nutrient limitation in the phytoplankton populations was estimated utilizing the N: P ratio since the phosphate concentration was similar at all stations. Lysogeny was detected in offshore populations that were nitrogen-limited as determined by inorganic N: P ratio. This relationship was not observed for the estuarine samples. Inorganic nutrient levels alone may give an incomplete indication of nutritional status of the population. The completed sequence of *Synechococcus* WH8102 has indicated the presence of genes for utilization of organic nitrogen sources, as well as genes for utilization of alternate sources of phosphorus (Palenik et al., 2003a). If these genes are common in *Synechococcus*, assessment of relative nutrient limitation in natural populations will be more difficult, especially in more productive estuarine environments.

The *Synechococcus* abundances at most of the cruise stations were higher than their corresponding cyanophage. This is most likely an under-reporting of the true cyanophage abundance as an artifact of the MPN method. A comparison of the MPN method of enumeration of viruses with both fluorescence microscopy and plaque assay methods has demonstrated that the MPN method gives an overall viral abundance lower than microscopy and higher than plaque assay due to the detection of host-specific and infective viruses only. However, all three methods demonstrated a similar level of precision (Cottrell and Suttle, 1995a).

The MPN method is very useful in the evaluation of environmental samples because it can separate the cyanophage from the ambient viral community. Since the host range of cyanophages varies, the host selection will alter the estimated cyanophage titer (Marston and Sallee, 2003a; Suttle and Chan, 1994a). Previous experiments have shown that using a phycoerythrin-containing host organism, which was isolated from an offshore environment and has been maintained in laboratory culture free from virus pressure for several years, will yield the highest cyanophage counts (Suttle and Chan, 1994a; Waterbury and Valois, 1993a). We used the isolate WH7803, which meets these criteria as well as being identified as an organism highly susceptible to a wide variety of cyanophages. In addition, we utilized our own isolate GM 9901, which is a phycoerythrin-containing marine GM 9901 isolate closely related to WH 7803 by *rbcl* phylogeny (data not shown). This isolate has consistently revealed a higher titer of cyanophage than WH7803 ($P=0.002$).

Multivariate statistical analysis is capable of ascertaining significant correlations based on combined factors, which might not be evident using either linear or multiple linear regressions (Clarke, 1988). The multidimensional pattern of the distribution of cyanophage induction can be depicted in a multi-dimensional scaling diagram (MDS plot). The separation between the oligotrophic, coastal, and eutrophic sampling sites can be readily discerned (Fig. 5-9). The distribution of cyanophage induction can then be compared to all measured environmental parameters, both separately and in combination with each other to determine correlations between cyanophage induction and the environmental variables.

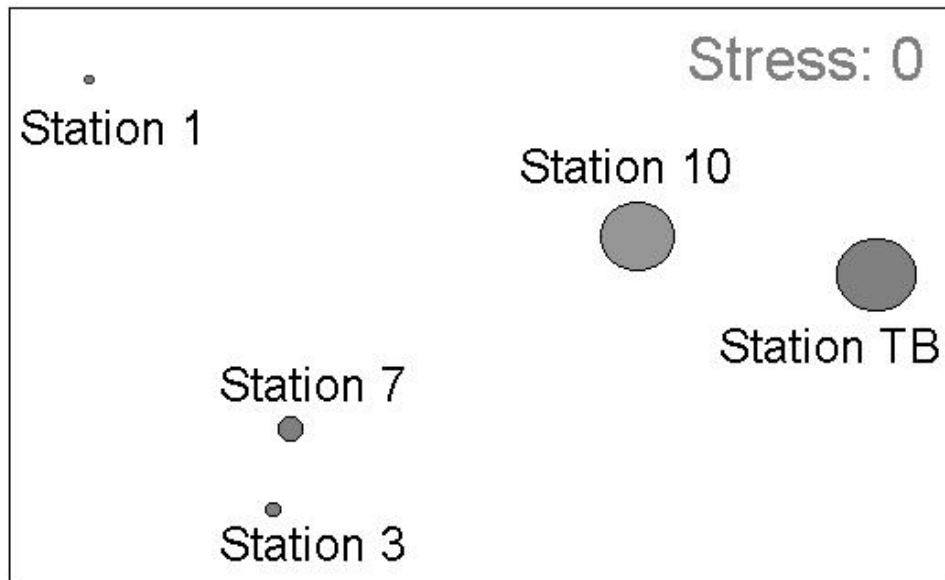


Figure 5-9: Multidimensional scaling plot (MDS) of the level of cyanophage induction from all experiments performed. The circles denote the ambient level of cyanophage.

Multivariate analysis indicated that there was a significant correlation between cyanophage induction and measured environmental variables ($\rho=0.818$, $P=0.007$). Further optimization of the analysis by utilizing transformed data indicated that the level of cyanophage induction, measured by both the parameters of cyanophage ml-1 and percentage change, were most closely and negatively correlated with the three combined parameters of temperature, *Synechococcus* abundance, and cyanophage abundance ($\rho=0.867$). The level of cyanophage induction was higher under the combined conditions of lower temperature, lower ambient *Synechococcus* abundance, and lower ambient level of cyanophage. There was insufficient data to analyze the contribution of ambient nutrient level to the observed distribution of cyanophage induction.

Re-analysis of the data from a yearlong seasonal study of cyanophage induction (McDaniel et al., 2002) utilizing the multivariate RELATE and BIOENV tests provided additional support for these conclusions. This analysis demonstrated that there was a significant relationship between the level of cyanophage induction and at least some of the measured environmental parameters ($P=0.01$). The best observed correlation was an inverse relationship between the level of cyanophage induction and ambient level of cyanophage ($\rho=-0.812$). Excellent correlation was also obtained between the level of proophage induction and the combined factors of ambient level of cyanophage and ambient level of *Synechococcus* ($\rho=-0.780$).

A recent study of the prevalence of lysogeny in heterotrophic populations from surface, mesopelagic and deep waters of the Mediterranean and Baltic Seas showed that the prevalence of lysogeny was inversely correlated with host abundance, followed by viral abundance and bacterial production (Weinbauer, Brettar, and Hofle, 2003). There was a similar relationship between the occurrence of lysogeny and primary productivity during the seasonal study of lysogeny in Tampa Bay (McDaniel et al., 2002).

The Gulf of Mexico cruise experiments also demonstrated an inverse correlation between primary productivity and level of cyanophage induction. However, the number of stations with

data for comparison was too small to determine statistical significance at a high confidence interval (Pearson correlation, $r = -0.9525$, $P = 0.14$, $n=3$).

These experiments have indicated that nutrient amendment alone is rarely sufficient to stimulate prophage induction in *Synechococcus*. In both coastal and oligotrophic environments, the ambient level of cyanophage and *Synechococcus* hosts, possibly in combination with temperature and level of productivity, that determines the prevalence of lysogeny and the level of prophage induction in natural populations of *Synechococcus*.

CHAPTER 6

Comparison of Lytic and Temperate Cyanophages of Marine *Synechococcus*.

CHAPTER SUMMARY

Lytic cyanophages of picoplankton are abundant, widespread, and readily isolated from seawater samples. The myovirus morphotype is the most common type of lytic phage isolated from *Synechococcus* and has a wide host range, occasionally even infecting other cyanobacterial species. In contrast, podovirus and siphovirus morphologies have been isolated less frequently and podovirus types have demonstrated a narrow host range. TEM Examination of our collection of 35 infectious *Synechococcus* cyanophage isolates revealed 32 myoviruses and two podoviruses. These lytic viruses have been screened for cross-infectivity against 26 *Synechococcus* isolates with percentage of successful *Synechococcus* lytic infections ranging from 0%, (host of isolation only) to 67%. *Synechococcus* cyanophages may contain a capsid protein gene analogous to the g20 gene in coliphage T4. This gene has been utilized in examination of the diversity of natural cyanophage communities. However, it has not been detected in all cyanophages and varies in abundance both temporally and spatially. A random screening of our cyanophage isolates has indicated that this gene was present in nine of 15 isolates examined to date (60%). In addition to lytic viral infection, natural *Synechococcus* populations have demonstrated prophage induction. In our *Synechococcus* isolates, eleven of 25 strains (44%) produced a statistically significant increase in virus-like particles in response to Mitomycin C. No correlation was found between the presence of cyanophage induction and the level of resistance to lytic infection. One of these *Synechococcus* phage-host systems has been studied in greater detail. In contrast to typical lytic cyanophages, the induced *Synechococcus* isolate produces non-tailed viral particles. The putative prophage has a very limited host range

and has been unable to infect any of the alternate hosts offered. Experiments with differential nucleic acid stains and S1 nuclease suggest that the phage contains single stranded DNA (ssDNA). Sequencing of the phage genome is in progress. Investigation of potential natural inducing agents revealed that high levels of light led to cyanophage induction in isolate P99-14, with VLP's produced at levels of $2-3 \times 10^7$ VLP's ml⁻¹. Treatment with heat, cold, and markedly decreased or increased salinity failed to cause cyanophage induction of natural *Synechococcus* populations.

INTRODUCTION

Marine *Synechococcus* are unicellular prokaryotic cyanobacteria that are a significant component of the marine picoplankton. An estimated 25% of worldwide primary productivity and up to 60% of the total primary productivity at the 1% light level are attributed to these organisms (Waterbury et al., 1986b). Because of their obvious ecological importance, a great deal of research has focused on the variables influencing the growth and productivity of marine *Synechococcus*. Cyanophages that infect marine *Synechococcus* strains are widespread and readily isolated from seawater. These phages have been confirmed to have both a high abundance and wide genetic diversity that varies both spatially and temporally [reviewed in (Mann, 2003b)]. An early experimental observation of natural populations of *Synechococcus* indicated that these picocyanobacteria were much less sensitive than other types of phytoplankton to the inhibitory effect of viral concentrates (Suttle, 1992). One possible explanation for this observed resistance could be the inherent immunity conferred by integrated prophage.

Lysogeny has been well documented in heterotrophic bacteria, in both cultured isolates and natural populations (Ackermann, 2003; Ackermann and DuBow, 1987; Cochran, Kellogg, and Paul, 1998; Jiang and Paul, 1996a). Fewer studies have been performed to date on the occurrence of lysogeny in autotrophic organisms. Early experiments demonstrated the presence of lysogeny in the freshwater cyanobacterium *Plectonema boryanum* (Rimon and Oppenheim, 1975), the marine filamentous form *Phormidium* (Ohki and Fujita, 1996), and in cultured and natural samples of the filamentous cyanobacterium *Trichodesmium* (Ohki, 1999).

In picoplanktonic cyanobacteria, induction of cyanophage has been demonstrated from a cultured marine *Synechococcus* species isolated from the coastal waters off Kyushu, Japan. In this case the cultured host was lysogenized by a marine phage isolated from natural samples. In the lysogenized host, prophage induction occurred in response to UV light, Mitomycin C (Sode et al., 1994a), and a heavy metal (Sode, Oonari, and Oozeki, 1997b).

Recently, studies have demonstrated that lysogeny occurs in natural populations of marine *Synechococcus* (McDaniel et al., 2002; Ortmann, Lawrence, and Suttle, 2002b). A seasonal pattern was observed in the detected inductions, with a cluster of positive inductions in late winter to early spring (McDaniel et al., 2002). Research on natural populations of *Synechococcus* in the Gulf of Mexico has shown that the primary factors influencing the prevalence and level of prophage induction in *Synechococcus* appear to be the ambient level of *Synechococcus*, cyanophage and possibly level of primary productivity (McDaniel and Paul, 2005). In this set of experiments, nutrient stimulation did not enable prophage induction but did on occasion reduce viral production.

Fragmentary preliminary evidence suggests that lysogeny in cyanobacteria may have a similar molecular basis to that of heterotrophic organisms (Owtrim and Coleman, 1987b). In the well-known lysogenic system of *Escherichia coli*/ λ , prophage induction can be precipitated by synthesis of a protein known as RecA, which responds to direct DNA damage. In this study, a *recA* homolog was isolated from the cyanobacteria *Anabaena variabilis*, which was able to restore spontaneous induction when introduced into *E. coli recA* deficient mutants

At this time it is unknown how widespread lysogeny may be in autotrophic prokaryotes. The factors controlling the switch from lysogenic to lytic existence in cyanophage are also currently unknown. The goals of this study were to investigate the prevalence of lysogeny in cultured *Synechococcus*, to examine the relationship between the level of resistance to lytic cyanophage infection and lysogeny, and explore potential triggers of prophage induction in marine *Synechococcus*.

MATERIALS AND METHODS

***Synechococcus* and Cyanophage Isolation, Cultivation, and Cross Infectivity**

Cyanophage isolates were obtained by serial dilution method using 0.2 μ m filtered natural seawater samples using host organism *Synechococcus* WH 7803. *Synechococcus* isolates were

also obtained by a serial dilution method with 1.0 μ m filtered seawater samples in SN media. All isolates obtained during this sampling were phycoerythrin-containing marine types classified as cluster 5.1 (formerly marine cluster A) isolates according to the most recent Bergey's classification (Herdman et al., 2001). These cultures are clonal, based on streak plating on solid media with single colony isolation. However, the *Synechococcus* cultures are not axenic despite repeated attempts with various methods of purification.

Viral cross infectivity/ host susceptibility experiments were performed in microtiter plates. Two hundred and thirty microliters of each host isolate were freshly diluted 1:10 with sterile SN media and inoculated with 20 μ l of viral lysate. Controls were prepared separately with a similar 1:10 dilution and amendment with 20 μ l of sterile media. Microtiter plates were grown at 26°C and 18-30 μ mol/m²s cool white fluorescent light. The estimated growth of the isolates was estimated by the density of the control cultures and placed in a growth category scored as light, moderate or heavy. The treatment cultures were observed for lysis (I=Immune, L=Lysed, P=Partially lysed). SYBR Gold staining confirmed presence of virus in the treatment wells.

Survey of Cyanophage Isolates for gp20 gene

Cyanophage isolates were screened for the presence of the capsid gene gp20 by Polymerase Chain Reaction (PCR). Random cyanophage isolates were selected for screening. Fifty microliter reactions were performed either with extracted cyanophage DNA or using crude viral lysate as per Marston and Sallee (2003). The primers utilized were designated CPS1 and CPS2. These primers amplify a 165-bp region of the g20 capsid protein gene. These primers have been observed to produce amplicon from the most divergent types of cyanophage (Marston and Sallee, 2003b).

The primer sequences were as follows: CPS1 (5' GTAGWATTTTCTACATTGAYGTTGG 3'); and CPS2 (5' GGTARCCAGAAATCYTCMAGCAT 3'). Reactions were performed in 50 μ l volumes with 5 μ l of 10x reaction buffer (Invitrogen, Carlsbad, Calif.), 2.5mM MgCl₂, 10 μ M concentration of each deoxynucleoside triphosphate (dNTP), 0.5 μ M concentration of each primer,

1.25U *Taq* DNA polymerase and 1 μ l of extracted viral DNA or viral lysate. Reactions were carried out in a Bio Rad MyCycler thermocycler with an initial denaturation step of 94°C for 2 minutes, followed by 32 cycles of 94°C for 1 min, 50°C for 1min and 72°C for 1min followed by a final extension of 72°C for 2 min. Products were examined on a 1.5% agarose gel. Each viral isolate was repeated at least twice to confirm the results of the PCR.

***Synechococcus* Prophage Induction**

All isolates were grown in SN media at 26°C and 18 $\mu\text{mol m}^{-2} \text{s}^{-1}$ cool white fluorescent light on a shaker at 150 rpm. Growth of the cultures was measured by A750. The cultures were divided into treatment and control samples when cultures reached mid log-phase (0.09-0.1). The treatment flasks were amended with 0.5 $\mu\text{g ml}^{-1}$ of Mitomycin C. Sub samples from both treatment and control flasks were removed and centrifuged at 10,000x g for 10 minutes followed by 0.2 μm filtration to remove the *Synechococcus* cells. Virus like particles (VLP's) were enumerated by SYBR Gold staining (Chen et al., 2001; Noble and Fuhrman, 1998).

Environmental Prophage Induction

Naturally variable environmental conditions were investigated to determine if they could be potential triggers of prophage induction in the environment. These included cold shock, heat shock, pulse of phosphate, high salinity (2x ambient) and decreased salinity (0.5x ambient). Negative control and positive control samples (Mitomycin C) were also measured. All experiments were conducted with triplicate natural seawater samples. The time of sampling was early spring (March), which has been previously discerned to be a likely time for *Synechococcus* prophage induction to occur.

Triplicate water samples were obtained from the eastern end of the St. Petersburg pier from sites approximately 50 meters distant from each other. The ambient temperature and salinity were measured and the samples taken to the lab for immediate processing. The samples were initially 3 μm filtered to remove grazing organisms. Next, three 200 ml replicate samples were prepared by the viral reduction method to decrease the ambient level of cyanophage, as

earlier described (Chapters 3 and 4). A 25 ml sub-sample of each replicate was then subjected to one of seven possible treatments: untreated negative control, Mitomycin C positive control, phosphate nutrient pulse, heat shock; cold shock, 0.5x salinity, and 2x salinity.

The negative control sample was viral reduced and returned to the original sample volume with virus-free seawater. The Mitomycin C positive control and phosphate treated samples were prepared similarly, then amended with $0.5 \mu\text{g ml}^{-1}$ Mitomycin C, or $10 \mu\text{M}$ phosphate (NaH_2PO_4) respectively. The heat treatment was prepared similarly to the negative control and then subjected to a temperature 20°C above ambient (38°C) in a water bath. The cold shock treatment was prepared in the same way with a 10°C decrease in temperature (8°C) in a separate water bath. The 0.5x salinity was brought to the correct salinity level using a mixture of virus-free seawater and sterile reagent-grade water ($S=0$). For the 2x salinity treatment, the sample was returned to its original volume with a combination of virus free seawater and sterile 4x concentrated minimal seawater (MgSO_4 , NaCl).

All of the samples were incubated in the dark for a total of 24 hours to allow response to the treatments. The number of cyanophage in each sample was then enumerated using serial dilution followed by addition of the host organism *Synechococcus* WH7803, followed by incubation and enumeration by the MPN method as previously described.

***Synechococcus* Prophage Induction with Light**

Synechococcus isolates were maintained at $18 \mu\text{mol m}^{-2} \text{s}^{-1}$ on a twelve-hour light/dark cycle using cool white fluorescent bulbs. Treatment cultures were shifted to either half, two times or four times this light intensity. In addition, the 4x treatment cultures were increased to a 24-hour light cycle. All cultures were grown in enriched natural seawater (SN) media and maintained at 26°C .

Lysed cultures were centrifuged at 9-10,000x g for 20 minutes and then $0.2 \mu\text{m}$ filtered to remove cell debris and residual *Synechococcus* cells. Viral abundances in the lysates were determined as previously described, using SYBR Gold staining. Host cell abundances were

determined from unfiltered cultures, using their natural fluorescence under epifluorescence microscopy with blue excitation as previously described (Vernet, Mitchell, and Holm-Hansen, 1990b).

Concentration of Viral Lysates and Nucleic Acid Extraction

Synechococcus isolate P99-14 was grown to log-phase and induced with light as described earlier. Because of the generally low level of viruses in the lysates compared to heterotrophic lysogens, large volumes of lysate were prepared (800-1200 ml). Initial attempts with large volumes in a single, aerated flask provided an even lower titer of phage. Therefore, cultures were grown in multiple 500 ml culture flasks and the induced lysates were pooled. All lysates were centrifuged and filtered to remove debris as above. The viral particles were precipitated using the polyethylene glycol (PEG) method of Sambrook with some modifications (Sambrook and Russell, 2001).

In brief, the lysate was DNase and RNase digested ($1 \mu\text{g ml}^{-1}$ final concentration for one hour) to remove any residual host nucleic acid. The lysate was then treated with 1 M NaCl and centrifuged a second time to remove any residual cell debris. Ten percent PEG 6000 was added to the lysate (w/v) and it was precipitated on ice overnight. The lysate was then centrifuged at $9500 \times g$ for 20 minutes at 4°C to precipitate the viral particles. The supernatant was carefully aspirated. The particles were resuspended in SN media and the PEG was extracted with an equal volume of chloroform. Initial attempts to extract the nucleic acid were performed with a Wizard λ extraction kit (Promega Inc., Madison WI) followed by quantification with Hoescht fluorescent dye, utilized for quantification of dsDNA (Paul, 1982). These initial attempts failed to recover a quantifiable amount of DNA. The nucleic acid was subsequently extracted successfully from PEG concentrated lysates using an RNeasy miniprep kit per the manufacturers instructions (Qiagen, Valencia, CA).

Cesium Chloride Purification of Viral Particles

To obtain viral particles free from host cells or other contaminants, the PEG-precipitated lysate was purified by a cesium chloride (CsCl) density step gradient according to the general protocol of Sambrook et al (2001), with modifications (Sambrook and Russell, 2001). Because the induced cyanophage is of marine origin, the CsCl density solutions were prepared with sterile, artificial seawater instead of SM media. The three density solutions were allowed to equilibrate in temperature overnight after which the density was measured with hydrometers. The density gradient steps were layered in an ultracentrifuge tube in the order 1.7, 1.5, and 1.3. Cesium chloride was added to the precipitated viral lysates to prepare a CsCl solution with a final density of 1.15. This solution containing the concentrated viruses was then layered carefully over the prepared gradients giving a final volume of 11 ml in each tube. The gradients were placed in a Beckman model LE-80 ultracentrifuge using an SW40-Ti swinging bucket rotor. The gradients were centrifuged at 29,000 rpm (120,000 x g) for 2 hours at 18° C. Gradients were harvested by needle puncture from the bottom of the centrifuge tube. SYBR Gold staining was used to evaluate each fraction for the presence and number of viral particles. Induced cyanophage isolates were concentrated at the 1.3-1.5 density interface.

The nucleic acid from CsCl purified virions was extracted using a formamide extraction followed by ethanol precipitation according to the general protocol of Sambrook et al (2001) with modifications (Sambrook and Russell, 2001). The tube containing the purified viral particles was amended with 1 volume of deionized formamide (Sigma, 99.5% minimum concentration), 0.1 volume of 2 M Tris, 0.5 volumes of 0.5 M EDTA (pH 8.0), and placed in a 65° C water bath for 30 minutes to rupture the capsids and release the nucleic acid. The DNA was precipitated with 6 volumes of 100% ethanol (room temperature) followed by a brief centrifugation. The supernatant was discarded and the DNA redissolved in 300 µl of TE buffer. The DNA was reprecipitated with 6 µl of 5 M NaCl and 750 µl of 100% ethanol. The tube was centrifuged briefly as above and the supernatant discarded. The DNA was resuspended in 300 µl of TE buffer, as previously and stored at -80° C. The resuspended nucleic acid was quantified by Oligreen fluorescence.

Quantification of Nucleic Acid

Initial attempts to quantify the extracted nucleic acid were performed by comparing the sample to a standard curve of known quantity using Hoescht dye fluorescence. These attempts revealed either no nucleic acid in the samples or a small amount at the lower detection limit. Quantification was then attempted with the RiboGreen RNA quantitation assay (Molecular Probes, Eugene, OR). This assay is similar to the Hoescht assay in that it compares the fluorescence yield of a bound nucleic acid dye in an unknown sample to that of a standard curve. However, this assay is not specific to RNA alone. DNase treatment of the samples is required before quantification to make the assay specific for RNA.

Comparison by DNase and RNase digestion revealed that the extracted viral nucleic acid was DNA rather than RNA. Further quantification was then performed utilizing the OliGreen ssDNA quantitation assay (Molecular Probes, Eugene, OR). Similar to the other protocols, bound OliGreen dye allows determination of the amount of ssDNA in an unknown sample by comparison to a standard curve with a known quantity of nucleic acid. In brief, an oligonucleotide dilution series is prepared in triplicate in 1X TE buffer with a concentration range from 100 pg ml^{-1} to 50 ng ml^{-1} with a ssDNA oligonucleotide standard provided by the manufacturer. One milliliter of the diluted OliGreen dye prepared as per manufacturer's instructions is placed in each standard curve sample and unknown sample. The fluorescence yield of each sample is measured on a spectrofluorometer (Excitation 480 nm, Emission 520 nm). Unknowns are then quantified using a standard linear regression of the standard curve samples.

S1 Nuclease Digestion

Nuclease S1 degrades single-stranded nucleic acid, both RNA and DNA but preferentially DNA. Double-stranded nucleic acids are resistant to the enzyme, except at very high concentrations (Sambrook and Russell, 2001). This nuclease was utilized to characterize the nucleic acid of the induced cyanophage from isolate P99-14. Digestions were prepared

according to the stated activity of the enzyme as one unit of nuclease S1 to digest 100 ng of ssDNA in 10 minutes at 37° C (Promega, Madison, WI). The nucleic acid of the induced cyanophage was digested as specified with Phi X174 ssDNA (New England Biolabs, Beverly, MA), and Lambda dsDNA (Promega, Madison, WI) digested in parallel as controls. Each digested nucleic acid and an undigested control was quantified in duplicate by Oligreen fluorescence at the start of the experiment, and at 10 and 30 minutes.

Enumeration and Examination of Viruses by SYBR and DAPI Staining

SYBR Gold stain, a proprietary formula with an undisclosed mode of action, was performed as previously described. This stain is known to bind and fluoresce under blue excitation in response to all types of nucleic acid including, double-stranded DNA (dsDNA), single-stranded DNA (ssDNA) and RNA (Noble, 2001).

In contrast, the nucleic acid stain DAPI (4'6-diamidino-2-phenylindole) binds to A-T rich sequences in the major groove of DNA, being specific to dsDNA (Howard-Jones, Frischer, and Verity, 2001). For DAPI staining of viral particles 5ml samples were prepared as for SYBR staining, at the appropriate dilution and fixed with 1% 0.02 µm filtered formalin. Fifty microliters of the 10⁻³ M stock solution was added to the sample for a final concentration of 10⁻⁵ M. The samples were incubated in the dark for one hour. Three 1 ml sub samples from each sample were filtered through an Anodisc 0.02 µm filter and placed on a glass slide. Each filter was covered with one small drop of immersion oil and a coverslip. Each slide was viewed by epifluorescence microscopy using an Olympus BX-60 microscope with UV excitation (WU filter).

Transmission Electron Microscopy

TEM images of the induced cyanophage were obtained by spotting a small drop of 0.2 µm filtered, ultracentrifuge concentrated or polyethylene glycol precipitated cyanophage lysate onto a Formvar coated grid. The viruses were allowed to adsorb to the grids for several minutes and the excess liquid wicked off with filter paper. The grids were allowed to air dry followed by

negative staining with uranyl acetate for 30 to 60 seconds. After the grids were dry, they were immediately viewed with a Hitachi 7100 electron microscope.

Cultures of *Synechococcus* isolate N were observed in time series during a high light intensity prophage induction experiment. To prepare the samples for microscopy, a 20 ml sub sample of inducing *Synechococcus* culture was removed at each sampling time point and pelleted in a centrifuge at 9000xg for 20 minutes. The pellet was re-suspended in 2 ml 75% sterile seawater or SN media. The sample was fixed with 18ml glutaraldehyde fixative stock (1% final concentration) overnight at 4° C. After the cells were fixed, the sample was pelleted at 9,000-9,500x g for 5-10 minutes, and the fixative gently aspirated. The pellet was resuspended and washed gently with 1 ml seawater and transferred to a 1.5 ml microcentrifuge tube and incubated for 10-15 minutes. The sample was pelleted again and the seawater gently aspirated. The sample was embedded in 0.5-1 ml of molten 1% agar and immediately centrifuged at 9,000x g for 30 seconds. The sample was cooled to harden the agar at 4° C. One millimeter sections of the agar embedded sample were then prepared by en-bloc staining with uranyl acetate, 2% Osmium tetroxide, followed by alcohol/acetone dehydration and infiltration with Spurr's resin. Ultra thin sections were observed as above.

Statistical Analyses

The level of viruses in the treatment and control cultures were compared by paired t-test using Minitab statistical software, release 13. Multivariate statistical analyses of the pattern of susceptibility of the *Synechococcus* host organisms and other variables were performed using the ANOSIM algorithm in the Primer statistical software (Primer-E Ltd., Plymouth Marine Laboratory, U.K. www.primer-e.com). Analyses between lysogeny and resistance to infection were performed using both standard chi-square analysis (Minitab) and multivariate ANOSIM analysis (Primer).

RESULTS

Twenty-five *Synechococcus* and 35 *Synechococcus* cyanophage isolates were obtained from natural seawater samples during a research cruise in the Gulf of Mexico during the summer of 1999. All of the cyanophages were tested against all of the *Synechococcus* isolates to determine the level of cross-infectivity among the cyanophages and to determine if some of the hosts might demonstrate homoimmunity conferred by the presence of integrated prophage.

The survival rates of the host organisms ranged from 11% to 100%. The cyanophages also varied in their level of virulence with some phages lysing none of the hosts to as many as 67% (Table 6-1). Statistical analysis found no relationship between whether the phage was isolated in the surface waters or at depth, or near-shore or offshore and its level of infectivity.

Cyanobacterial Isolate Designation.	Growth Rate	Virus																																			% Homoimmune		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35			
P99-01	heavy	I	I	I	I	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	11	
P99-02	heavy	I	I	I	I	L	L	I	L	L	P	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	17	
P99-03	moderate	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	all	
P99-04	heavy	I	I	I	I	I	I	I	P	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	29	
P99-05	moderate	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	P	I	L	I	P	I	I	P	L	P	L	L	I	I	I	I	I	I	I	91	
P99-06	moderate	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	L	P	I	I	I	I	I	I	I	I	I	I	97	
P99-07	moderate	I	I	I	L	I	I	I	I	I	I	L	I	L	I	I	I	I	I	I	L	P	L	P	P	L	I	I	I	I	I	I	I	I	P	P	P	83	
P99-08	light	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	P	I	L	P	P	P	I	I	I	I	I	I	I	I	I	I	P	I	P	97	
P99-09	light	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	P	P	P	P	P	P	P	I	I	I	I	I	I	I	I	all	
P99-10	light	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	P	I	P	P	P	I	I	I	I	I	I	I	I	I	I	I	I	I	all	
P99-12	heavy	I	I	I	L	I	P	I	I	I	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	77	
P99-13	moderate	I	I	I	L	P	P	I	P	I	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	29	
P99-14	heavy	I	I	I	P	L	L	L	P	I	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	14	
P99-15	heavy	I	I	I	L	L	L	I	L	I	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	17
P99-16	moderate	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	all	
P99-17	heavy	I	I	I	L	P	L	I	L	P	I	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	20	
P99-18	heavy	I	I	I	I	I	I	I	I	I	L	I	I	I	I	I	I	I	I	L	L	L	P	L	L	L	L	L	L	L	L	L	L	L	L	L	L	43	
P99-19	heavy	I	I	I	L	I	P	I	I	I	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	26	
P99-20	heavy	I	I	I	L	L	L	I	P	P	I	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	23	
P99-21	light	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	all	
P99-22	heavy	I	I	I	L	I	L	I	I	I	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	23	
P99-23	light	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	91	
P99-24	light	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	all	
P99-25	heavy	I	I	I	L	I	P	I	I	I	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	26	
Control(WH 7803)	heavy	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	0	
Percent lysed		0	0	0	38	21	29	8	25	8	13	54	46	54	46	46	54	54	58	58	58	63	58	67	63	58	58	58	54	54	54	54	54	54	54	54	54		

Table 6-1: Cross-Infectivity of *Synechococcus* isolates with lytic cyanophage isolates. I=Immune to infection, L=Lysed, P=Partially, but not completely lysed.

Analysis of the cross infectivity data revealed that growth rate category is an important factor determining the susceptibility of a *Synechococcus* strain to a lytic cyanophage. A statistically significant relationship was observed, with isolates placed in rapid growth category were observed to be more susceptible ($R=0.58$, $P=0.001$).

Another interesting correlation was observed between infectivity and station of isolation. Cyanophages from station six were the least virulent and those from station 15 were the most virulent. The multidimensional scaling diagram (MDS) is a graphical representation of the three-dimensional groupings formed by the cyanophages based on their level of infectivity (Fig. 6-1). The reason for this pattern of infectivity is not immediately apparent. Both Station 15 and Station 6 were oligotrophic, offshore stations in the Gulf of Mexico. The obviously contrasting environment was a coastal station (number 13) and the viruses isolated from that station demonstrated an intermediate level of infectivity.

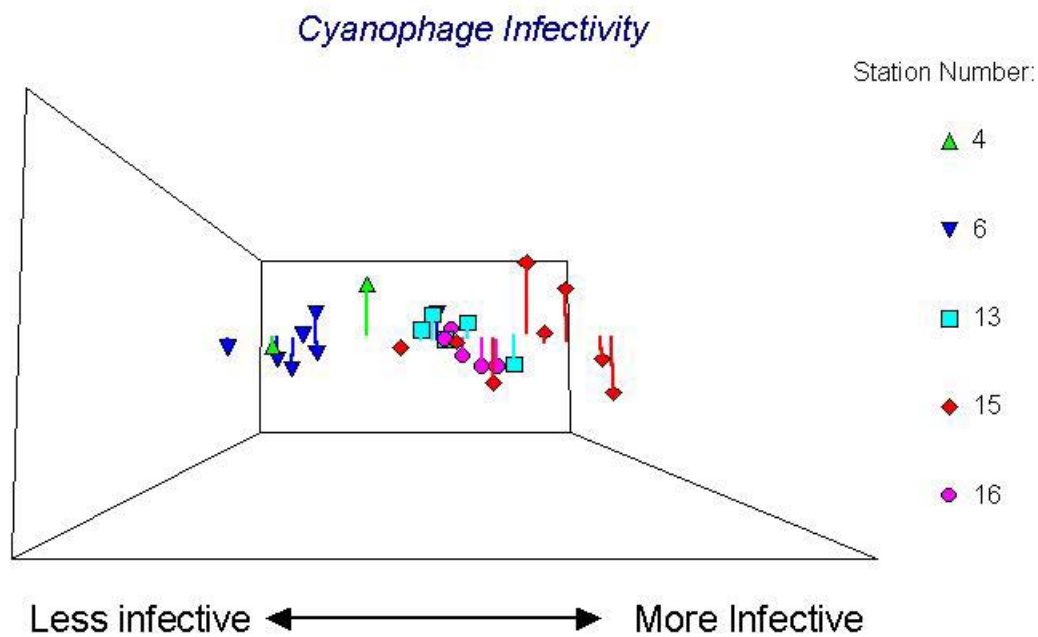


Figure 6-1: MDS diagram of the level of infectivity of cyanophage isolates. The cruise station of isolation is indicated by the symbol noted in the legend. Note the isolates from Station 15 are the most infective (red diamonds), and those from Station 6 are the least infective (blue triangles). Isolates from Station 4 (green triangles) have a wide range of infectivity.

Examination of the cyanophage isolates using TEM revealed that 33 of 35 cyanophage isolates examined to date are of the myovirus type. Some representative examples may be seen in figure 6-2. Prior researchers also reported a predominance of tailed cyanophages (Lu, Chen, and Hodson, 2001; Suttle, 2000). This tendency may be a bias due to the isolation procedure and frequent utilization of host WH7803 for isolation of cyanophages.

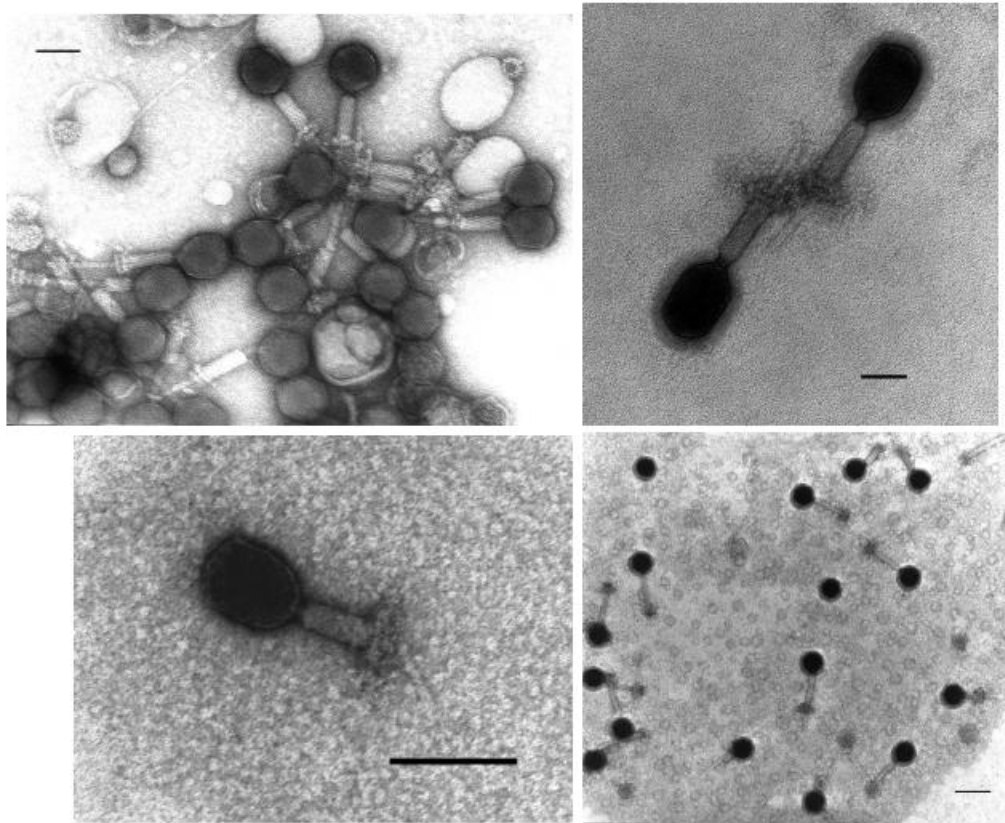


Figure 6-2: Some examples of lytic *Synechococcus* cyanophages.

A survey of the cyanophage isolates was performed to determine the prevalence of the gene for the capsid protein gp20, which is analogous to the gene found in coliphage T4 and is commonly found in *Synechococcus* cyanophages (Hambly et al., 2001). Fifteen of the 35 cyanophage isolates have been screened by PCR with cyanophage specific g20 primers. Of those 15, only nine were found to contain the gene (60%). This result is consistent with the observed prevalence of the gene in a year-long investigation of cyanophage diversity in a Rhode Island coastal area (Marston and Sallee, 2003b). Alternatively, the gene may be present in all

cyanophage isolates, but the primer set or PCR conditions require further refinement. In either case, it is clear that the gene for capsid protein gp20 is of marginal utility at this time for determining the phylogenetic relationships among *Synechococcus* cyanophages.

All 25 of the *Synechococcus* isolates were screened for the presence of prophage induction using Mitomycin C, a potent inducing agent. Initially, seven of the isolates demonstrated a statistically significant ($P < 0.05$) increase in viruses in response to Mitomycin C. An additional four isolates showed an increase in viruses that was significant at the greater than 90% confidence interval (Fig. 6-3). Repeated experimentation has verified these isolates demonstrate prophage induction (Table 6-2). A total of 44% of the *Synechococcus* isolates demonstrated prophage induction. Thus, a relatively high proportion of *Synechococcus* isolates appear to be lysogenic based on the presence of prophage induction in cultured isolates.

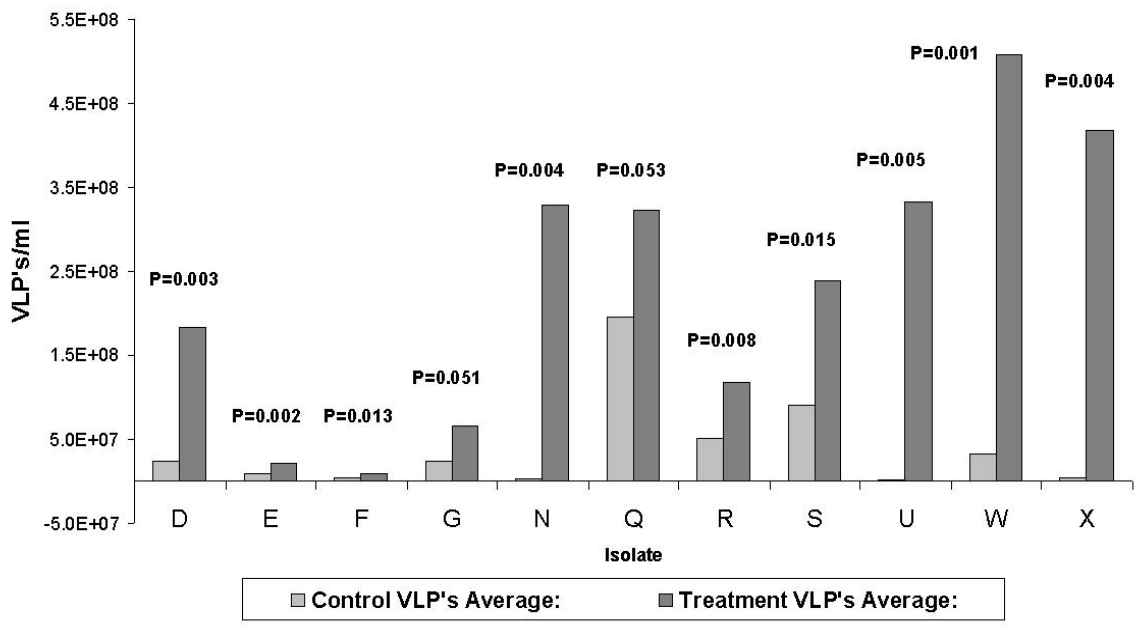


Figure 6-3: Level of treatment and control VLP's in eleven of 25 *Synechococcus* isolates that demonstrated a statistically significant level of prophage induction in response to Mitomycin C.

Isolate:	D	E	F	G	N	Q
Treatment VLP's	1.1E+09	6.4E+07	1.5E+06	6.9E+07	1.5E+08	2.8E+08
Experiment 1	1.1E+09	1.7E+08	3.1E+06	2.4E+07	2.2E+08	3.5E+08
	1.4E+09	1.8E+08	4.0E+06	2.0E+07	4.2E+08	3.4E+08
Average:	1.2E+09	1.4E+08	2.9E+06	3.8E+07	2.6E+08	3.2E+08
Control VLP's	9.9E+07	3.5E+07	1.4E+06	6.6E+06	2.8E+07	2.0E+08
Experiment 1	1.9E+07	4.5E+07	1.4E+06	2.9E+06	8.1E+07	2.7E+08
	9.2E+07	5.4E+07	2.3E+06	4.7E+06	3.0E+07	1.2E+08
Average:	7.0E+07	4.5E+07	1.7E+06	4.7E+06	4.6E+07	2.0E+08
P Value	0.005	0.052	0.077	0.078	0.0277	0.053
Treatment VLP's	1.9E+08	5.1E+07	4.6E+06	5.7E+07	3.3E+07	
Experiment 2	2.0E+08	4.8E+07	4.3E+06	7.7E+07	3.7E+07	
	1.6E+08	5.1E+07	3.5E+06	6.5E+07	3.3E+07	
Average:	1.8E+08	5.0E+07	4.1E+06	6.6E+07	3.4E+07	
Control VLP's	3.0E+07	2.4E+07	2.1E+06	4.3E+07	2.7E+06	
Experiment 2	2.0E+07	2.6E+07	3.5E+06	1.6E+07	2.1E+06	
	2.4E+07	2.9E+07	2.2E+06	1.4E+07	8.3E+05	
Average:	2.5E+07	2.6E+07	2.6E+06	2.4E+07	1.9E+06	
P Value	0.003	0.002	0.05	0.051	0.001	
Treatment VLP's	2.8E+08	2.1E+07	7.4E+06		2.9E+08	
Experiment 3	3.2E+08	2.2E+07	9.7E+06		3.5E+08	
	4.1E+08	2.3E+07	8.8E+06		3.4E+08	
Average:	3.4E+08	2.2E+07	8.6E+06		3.3E+08	
Control VLP's	1.3E+07	7.4E+06	4.0E+06		3.4E+06	
Experiment 3	1.4E+07	8.9E+06	5.1E+06		4.2E+06	
	1.3E+07	1.2E+07	6.2E+06		2.9E+06	
Average:	1.3E+07	9.3E+06	5.1E+06		3.5E+06	
P Value	0.006	0.002	0.013		0.004	
Isolate:	R	S	U	W	X	
Treatment VLP's	6.0E+08	1.9E+07	1.3E+07	2.6E+07	3.8E+08	
Experiment 1	1.1E+09	3.0E+07	1.8E+07	1.6E+07	2.3E+08	
	1.5E+09	2.2E+07	2.0E+07	4.2E+07	6.5E+08	
Average:	1.1E+09	2.4E+07	1.7E+07	2.8E+07	4.2E+08	
Control VLP's	3.3E+08	5.4E+06	1.7E+06	1.3E+06	2.8E+06	
Experiment 1	4.8E+08	1.4E+07	1.7E+06	6.6E+05	1.8E+06	
	6.6E+08	4.7E+06	9.7E+05	2.5E+06	8.5E+06	
Average:	4.9E+08	7.9E+06	1.5E+06	1.5E+06	4.4E+06	
P Value	0.008	0.004	0.009	0.031	0.004	
Treatment VLP's	9.8E+07	2.2E+08	7.5E+06	1.4E+08	1.1E+09	
Experiment 2	1.3E+08	2.5E+08	6.3E+06	1.8E+08	1.2E+09	
	1.3E+08	2.5E+08	1.1E+07	1.5E+08	1.4E+09	
Average:	1.2E+08	2.4E+08	8.3E+06	1.6E+08	1.2E+09	
Control VLP's	4.8E+07	1.2E+08	3.1E+06	3.6E+07	1.0E+07	
Experiment 2	4.9E+07	9.1E+07	2.5E+06	4.1E+07	1.3E+07	
	5.4E+07	6.2E+07	3.2E+06	4.2E+07	1.3E+07	
Average:	5.0E+07	9.0E+07	2.9E+06	3.9E+07	1.2E+07	
P Value	0.008	0.015	0.027	0.009	0.003	
Treatment VLP's	1.7E+08	7.5E+07	3.6E+08	4.8E+08	2.6E+09	
Experiment 3	1.5E+08	9.0E+07	2.7E+08	5.1E+08	1.9E+09	
	1.7E+08	9.0E+07	3.7E+08	5.3E+08	2.1E+09	
Average:	1.6E+08	8.5E+07	3.3E+08	5.1E+08	2.2E+09	
Control VLP's	9.2E+07	3.9E+07	3.0E+06	3.0E+07	2.0E+07	
Experiment 3	1.1E+07	5.3E+07	1.9E+06	3.0E+07	1.9E+07	
	8.4E+07	5.5E+07	2.7E+06	4.0E+07	1.2E+07	
Average:	6.2E+07	4.9E+07	2.6E+06	3.3E+07	1.7E+07	
P Value	0.023	0	0.005	0.001	0.004	

Table 6-2: Repeat screenings of *Synechococcus* isolates for the presence of prophage induction. bold script indicates a treatment VLP's significantly greater than the control count at a 95% confidence interval. Regular script indicates a 90% confidence interval.

Synechococcus isolate P99-14 (Isolate N) was initially selected for further experimentation due to its high level of prophage induction as well as its generally robust growth rate. Replicate experiments with Mitomycin C have consistently demonstrated a statistically significant increase in VLP's in comparison to controls. However, it was observed that the morphology of the induced cyanophage, when characterized by TEM, is quite different from most lytic cyanophages. In contrast to all of the lytic cyanophages examined in this study, the induced cyanophage appears to be a non-tailed virus. It appears to be spherical or polyhedral in shape and approximately 50nm in diameter (Fig. 6-4). The viral particle was initially prepared for TEM by ultracentrifugation. Additional observations of the virus were prepared by chemical precipitation to rule out the possibility that tail fibers had been broken off by the force of the centrifugation. Both methods confirmed the virus to be generally spherical in shape. Some views suggest possible icosahedral symmetry, but the level of resolution is insufficient to be definitive.

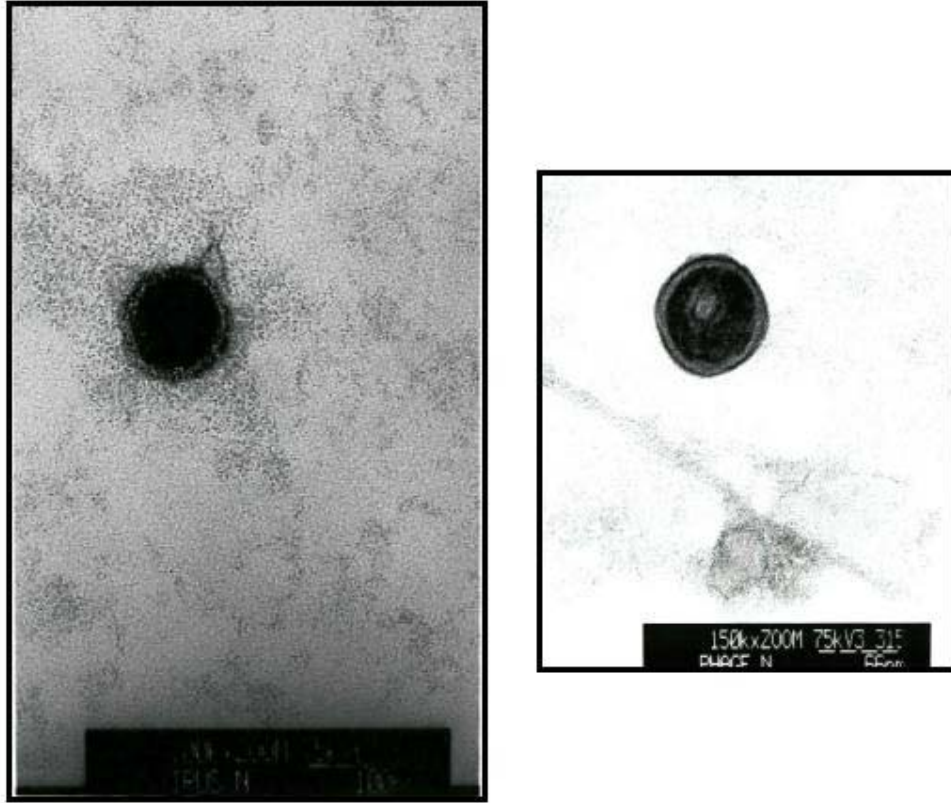


Figure 6-4: TEM images of the induced cyanophage from isolate P99-14. The phage on the left was concentrated by PEG precipitation. The phage on the right was concentrated by ultracentrifugation. Note the spherical shape and lack of a tail structure. The particle on the left also has a suggestion of icosahedral symmetry.

Analysis by both standard parametric and non-parametric statistical techniques demonstrated no correlation between level of resistance to lytic cyanophage infection and the presence of prophage induction in an isolate. This finding is contrary to the hypothesis that lysogeny should confer increased resistance to the host because of lysogenic homoimmunity. The immunity to superinfection conferred by an integrated prophage is usually against similar phages and the induced virus of isolate P99-14 is quite dissimilar. Future examination of the other induced cyanophages will determine if the other induced phages are distinct from the lytic cyanophages.

In addition to its unusual morphology, the induced cyanophage from *Synechococcus* host P99-14 appears to contain an unusual type of nucleic acid. The viral particles stain readily with the proprietary cyanine dye SYBR Gold, which is known to stain all types of nucleic acid. However, the particles do not stain with DAPI, which is specific to dsDNA. Samples of lytic cyanophages were stained with DAPI and SYBR as a positive control and they were readily visible with either method.

The extracted nucleic acid does not readily quantify with Hoescht dye, a commonly used fluorescent dye for dsDNA quantification (Paul, 1982), but is detectable with both RiboGreen and OliGreen fluorescent dyes. Hoescht has a similar mechanism of action to DAPI, binding to A-T rich regions of dsDNA. In contrast, RiboGreen dye is used specifically for quantification of RNA and OliGreen is used for ssDNA. However, both cross-react with other types of nucleic acid. Although the extracted viral nucleic acid was readily extracted using RNeasy and quantified with RiboGreen, selective digestion with RNase and DNase demonstrated conclusively that the extracted nucleic acid was DNA (data not shown). These findings are consistent with the presence of ssDNA.

Digestion experiments were performed on the extracted viral nucleic acid with nuclease S-1, which preferentially degrades single-stranded DNA. The rate of degradation of the induced cyanophage by S-1 was compared to phi X174, a known ssDNA virus, and phage λ , a known dsDNA virus. The nucleic acids of the induced cyanophage and phi X174 were both rapidly degraded, while the phage λ DNA was virtually untouched (Fig. 6-5). This result strongly

supported the presence of a ssDNA genome in the induced cyanophage. A single-stranded DNA virus would be an unusual finding, although isometric, ssDNA bacteriophages have been described and are classified in the family *Microviridae* (van Regenmortel et al., 2000).

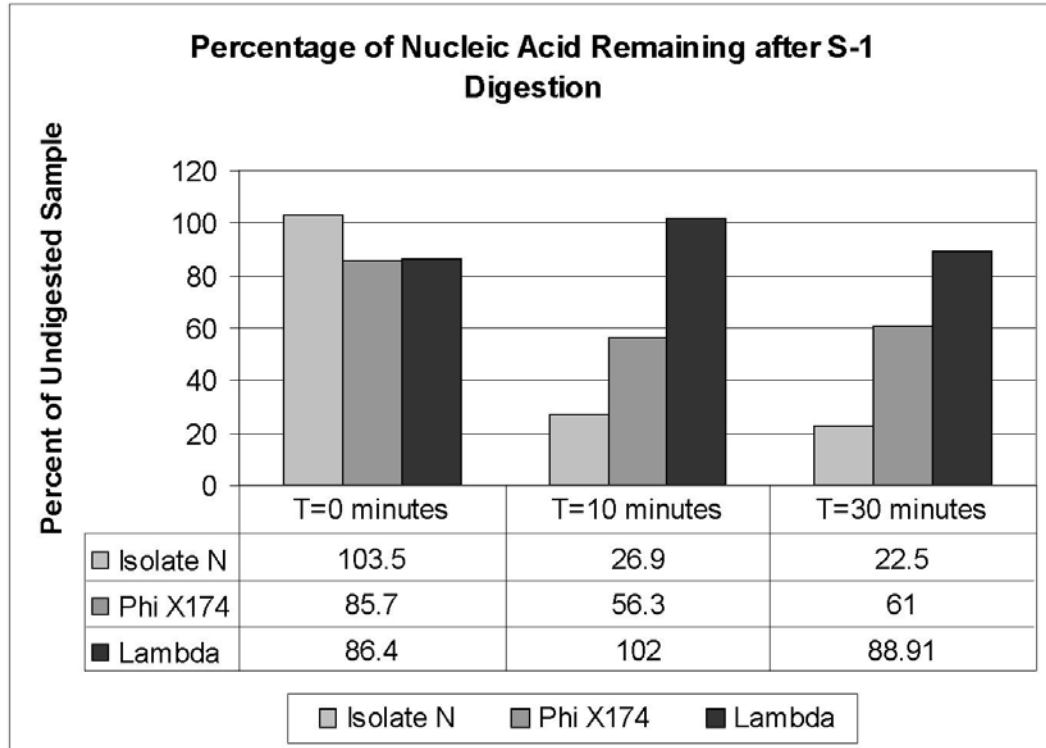


Figure 6-5: Comparative S1 nuclease digestion of extracted nucleic acid from induced cyanophage (Isolate N), Phi X174, and Phage λ . Results are expressed as the percentage of nucleic acid remaining after digestion in comparison to an undigested control.

Heterotrophic bacterial lysogens, such as *E. coli* / λ , typically produce around 10^{11} viruses per milliliter upon prophage induction. In contrast, *Synechococcus* isolate N consistently induces at a level of $2-3 \times 10^7$ VLP's per milliliter. Burst size estimates based on initial Mitomycin C induction experiments ranged from 15-31 VLP's/host. In addition, the low number of viruses has provided a challenge for further characterization of the putative lysogen due to the low yield of nucleic acid. After several attempts a sufficient quantity of CsCl purified viral particles was obtained and the nucleic acid was extracted.

The main concern in determining the nature of this viral particle is the fact that the cultures are not axenic. The possibility that this particle originates from a contaminant within the culture has not been conclusively confirmed. Bacterial contaminants from this isolate have been isolated separately on heterotrophic media and did not demonstrate prophage induction by treatment with Mitomycin C.

The genome of this viral particle is currently undergoing complete genome sequencing by collaborators. Analysis of the completed sequence should provide clarification about the nature of the particle and its functions. Additionally, genomic information will enable design of PCR primers and probes to decipher the location of the putative prophage in the host (chromosomally integrated or as a plasmid in replicative form). Such probes will also be useful in investigation of the prevalence of this form of lysogeny in the environment.

Another major goal of this study was to investigate prospective natural factors leading to prophage induction in *Synechococcus*. The occurrence of prophage induction in *Synechococcus* has been documented in the Tampa Bay estuary (McDaniel et al., 2002). Cyanophage induction experiments were performed on both natural samples and cultured isolates with variables that were observed to fluctuate within the marine environment in general, particularly in the Tampa Bay estuary. Because of the shallow depth in Tampa Bay, the water temperature can change relatively rapidly. There are high seasonal inputs of rain with extreme and rapid ranges in local salinity. Tampa Bay also has episodic inputs of nutrients from runoff associated with the heavy rainfalls. Thus, the variables investigated included high and low temperature, high and low salinity, phosphate pulses, and ranges in light level.

Investigation of natural populations of *Synechococcus* with the variables of high and low temperature stress, high and low relative salinity and phosphate pulse did not demonstrate any effect from these stressors. There was a statistically significant level of prophage induction in the positive control sample (Mitomycin C), indicating that prophage induction should have also occurred in response to an environmental factor that could cause prophage induction (Fig. 6-6).

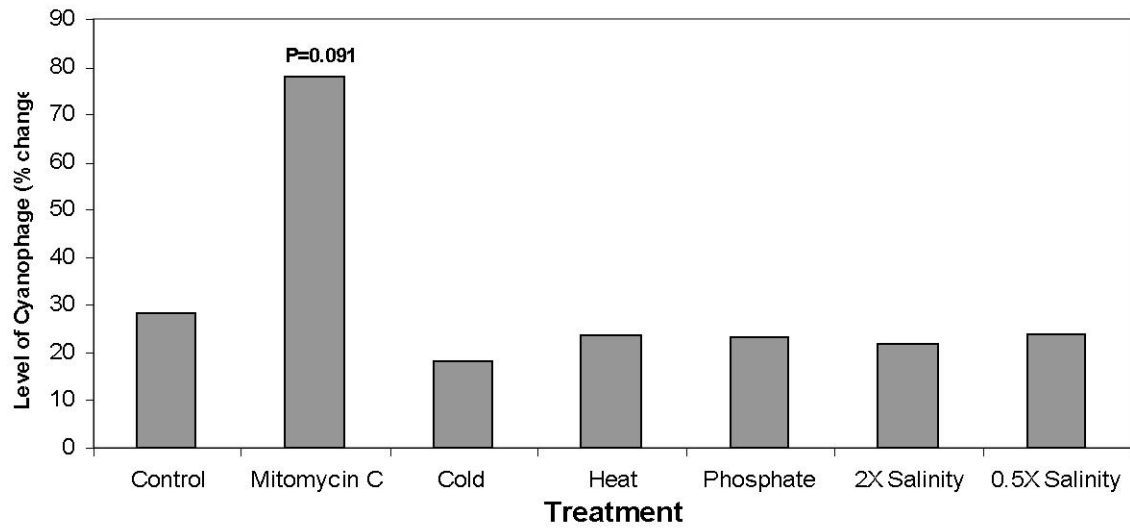


Figure 6-6: Prophage induction experiment with natural populations of *Synechococcus*. Results are the average of three replicate samples. Note the significant prophage induction in the Mitomycin C treated (positive control) sample. All other treatments were negative for an increase or decrease in cyanophage.

To test the effect of light on cyanophage prophage induction, experiments were performed with *Synechococcus* isolate P99-14, which is known to be inducible with Mitomycin C. The culture was grown using four differing light regimes including 1x (maintenance level used for cultures), 0.5x, 2x, and 4x.

Optimal growth of the host was observed at two times the maintenance light level. When the isolate was grown at four times the normal light level and increased to 24 hour exposure, cyanophage induction was observed. The P99-14 isolate consistently produces 3×10^7 VLP's ml⁻¹ in response to high light stimulus. This result strongly indicated that high light intensity might be an environmentally relevant trigger of prophage induction in *Synechococcus*.

Observation of isolate P99-14 undergoing induction by light demonstrated a progressive decrease in the number of host cells accompanied by an increase in VLP's (Fig. 6-7). This progressive lysis of the host cells is also observable visually (Fig. 6-8). Repeated experimentation has demonstrated that the maximum titer of viral particles was observed on the fifth day after shifting the rapidly growing isolate to high continuous light (data not shown).

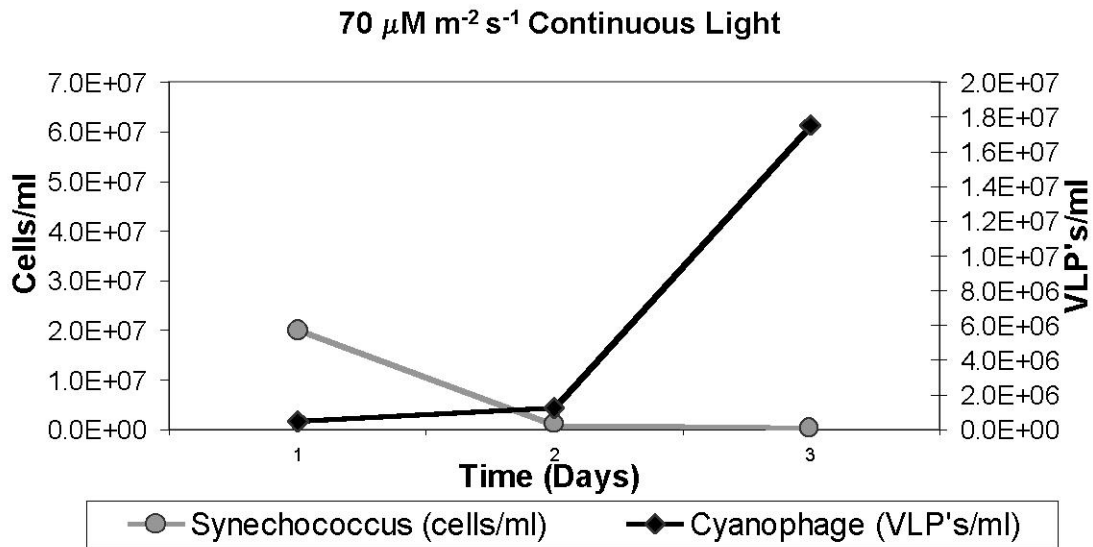
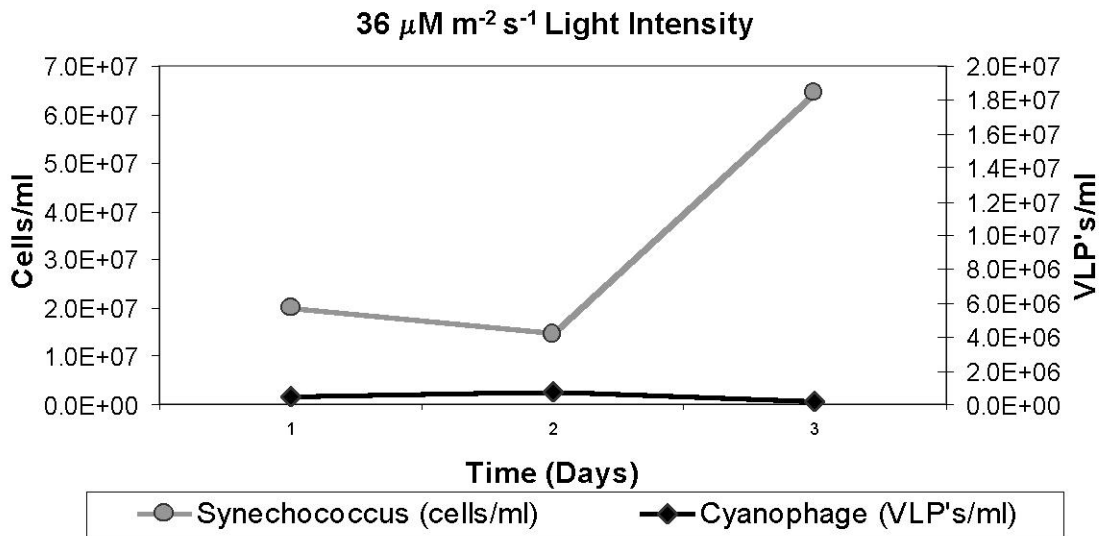


Figure 6-7: Prophage inductions of *Synechococcus* isolate P99-14 with light. The top figure is the abundance of host cells and VLP's at the 2x light level ($36 \mu\text{M m}^{-2} \text{s}^{-1}$). The bottom panel is the response of host cell abundance and VLP's to $70 \mu\text{M m}^{-2} \text{s}^{-1}$ continuous illumination. Note the rapid decrease in host abundance accompanied by a rapid increase in VLP's in the high light treated sample.

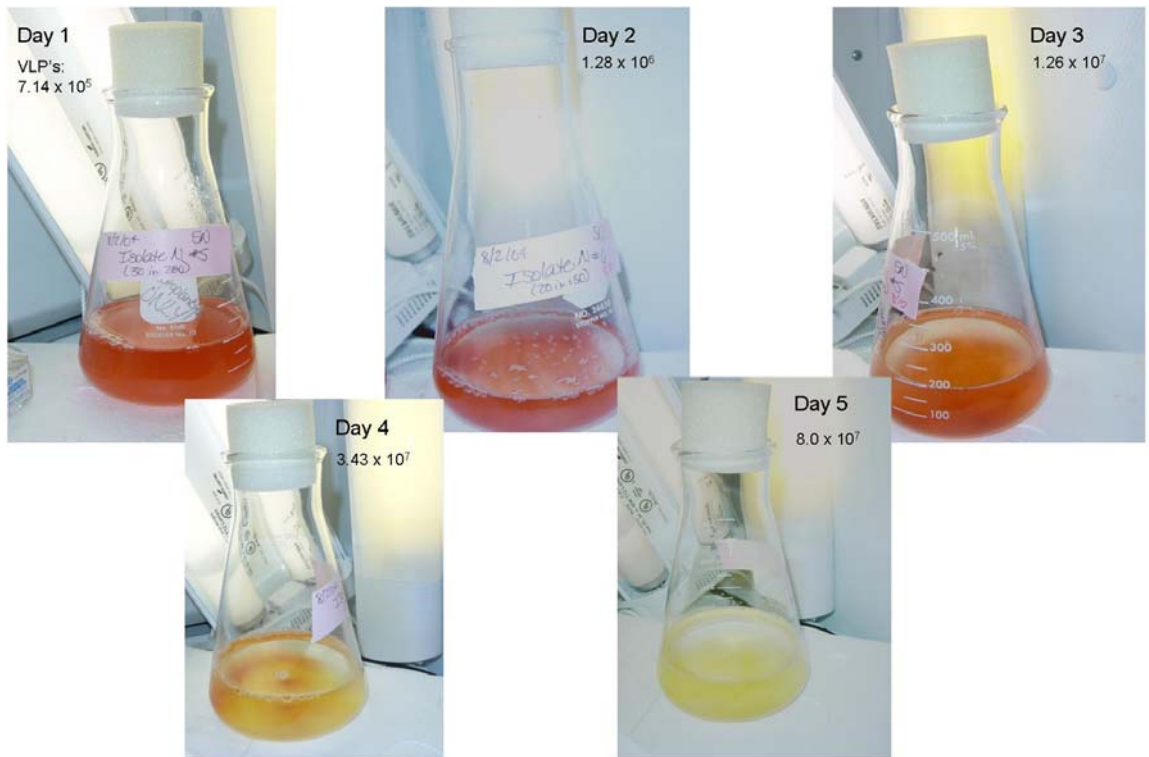


Figure 6-8: Photographs of an inducing culture of isolate P99-14 in response to elevated, continuous levels of light. Observe the progressive lysis of the culture. The indicated number value on each photograph is the number of VLP's ml^{-1} in the lysate at each time point.

The inducing *Synechococcus* culture was monitored by TEM. On the first day of light induction, the treatment cells appeared generally comparable to negative controls with some possible degradation of the cytoplasm. Viral particles were not yet observed (Fig. 6-9). By the second day of high light treatment some possible viral particles are observed in association with burst *Synechococcus* cells (Fig. 6-10). By the third day of high light, the thylakoid membranes had started to degrade. Larger numbers of ruptured cells and cell debris were observed (Fig. 6-11). Viral particles are not readily apparent within the remaining intact cells. Yet, the cytoplasm had a “grainy” appearance, which is potentially due to the differential density of viral particles. This general appearance is observed in the remaining intact cells throughout days four and five of the light induction (Fig. 6-12).

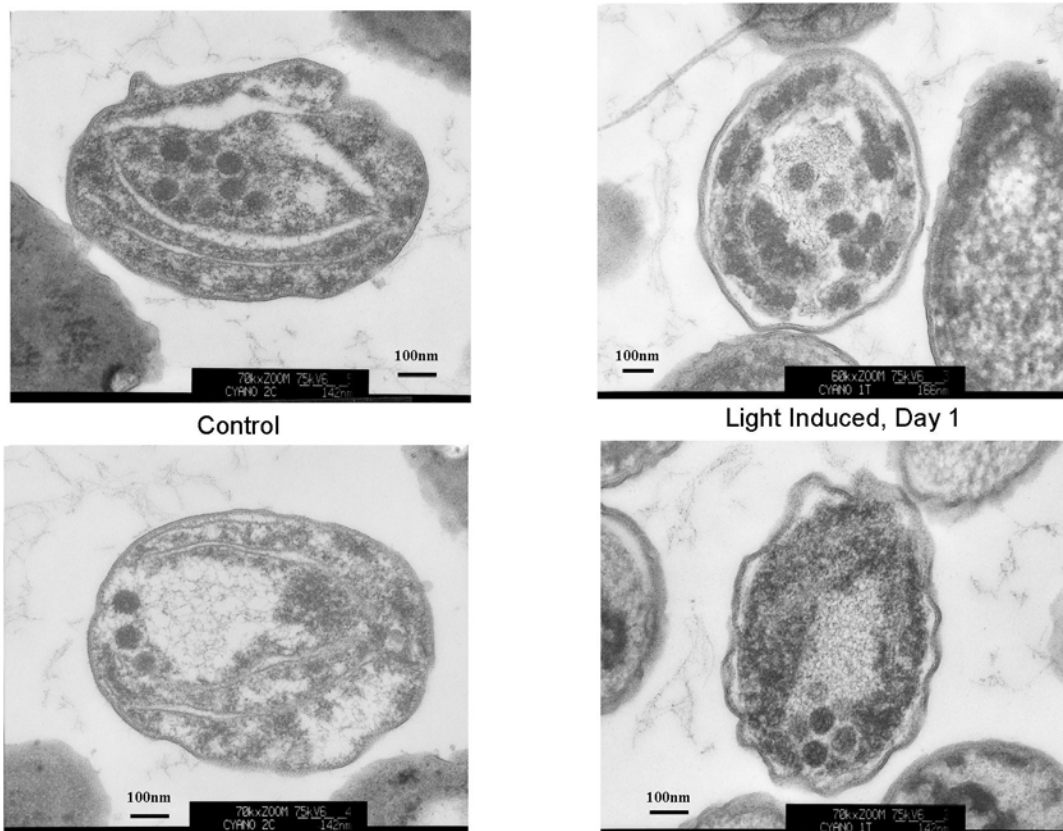
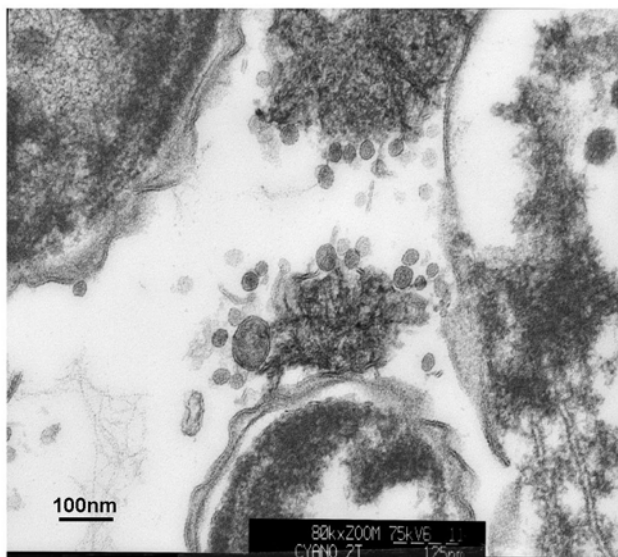


Figure 6-9: EM images of non-induced *Synechococcus* control culture and the light induced treatment culture after one day of exposure to high light levels. The large round dark cell inclusions are carboxysomes.



Light Induced, Day 2

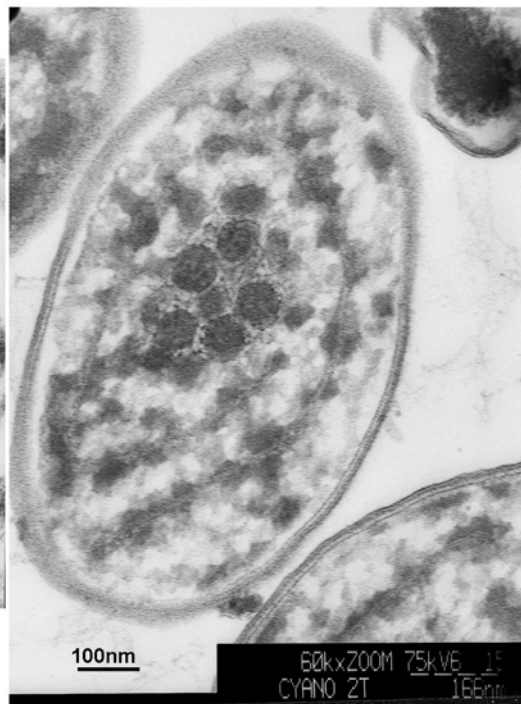
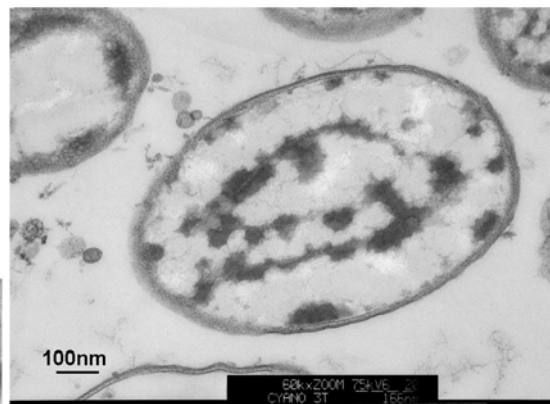
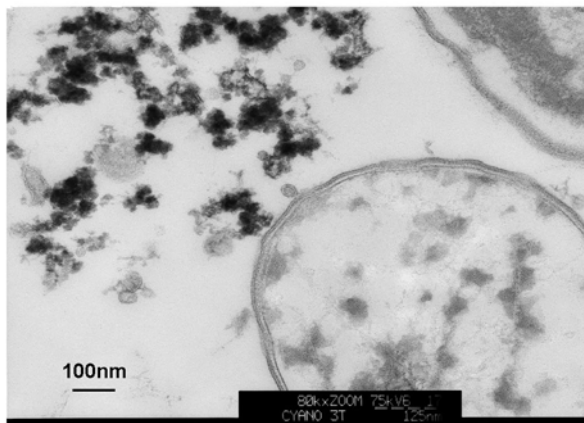


Figure 6-10: Light induced *Synechococcus* cells on day 2 of the treatment. Note the burst cells in the image on the left and the granular appearance of the cytoplasm of the intact cells on the right.



Light Induced, Day 3

Figure 6-11: On the third day of high light treatment large numbers of burst cells and cells with degraded thylakoids membranes are observed.

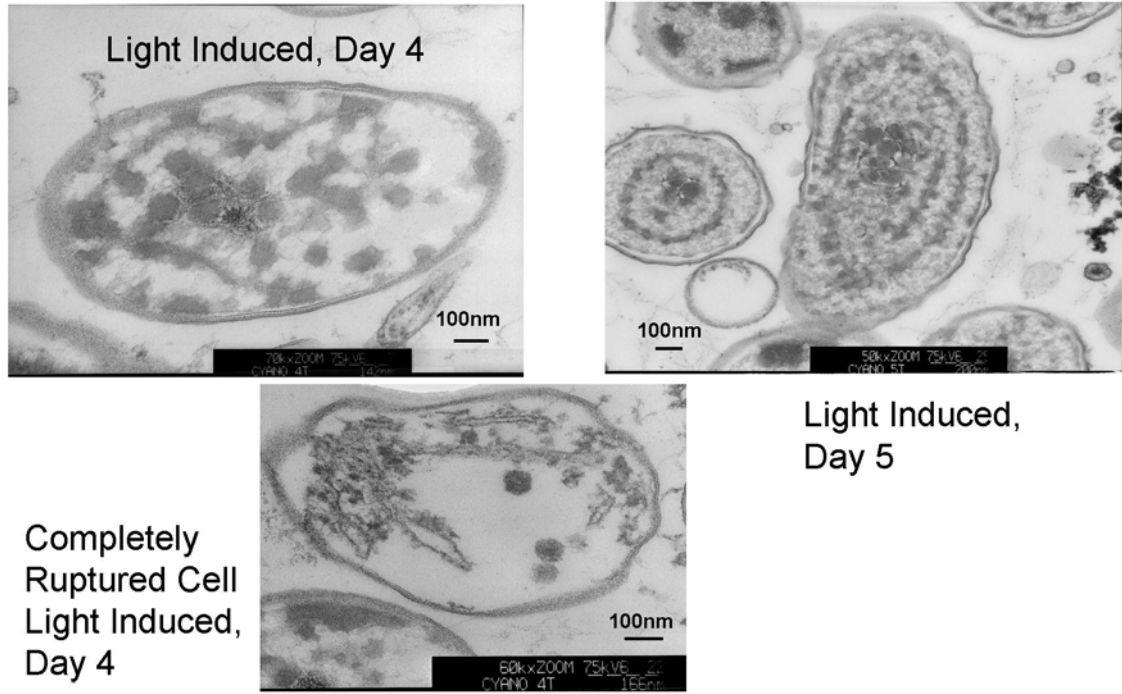


Figure 6-12: On the fourth and fifth day of high light treatment, burst cells continue to be frequently observed and the granular appearance of the cytoplasm is obvious in the remaining intact cells.

Discussion

Synechococcus cells have variable levels of resistance to infection and lytic *Synechococcus* cyanophages have a wide range of virulence. The growth rate of the *Synechococcus* host was observed to be an important factor in determining its susceptibility to infection. Not surprisingly, lytic viruses more readily infect rapidly growing hosts. The virulence of a viral isolate did not correspond to the general environment of isolation such as surface compared to 50m or coastal in comparison to oligotrophic. Lytic cyanophages isolated from certain cruise stations were clearly more infective than those from other stations but the reason for this was not obvious.

Prophage induction was present in 44% of *Synechococcus* isolates in culture, suggesting that *Synechococcus* prophage induction may be relatively common. No correlation was observed between resistance to lytic cyanophage infection and whether or not an isolate demonstrated prophage induction. However, ongoing analysis of one induced cyanophage indicates that inducible prophages may be quite different from common lytic cyanophages and may not provide immunity to them because immunity is generally only conferred to similar viral types.

Analysis of inducing cultures by TEM did not clearly demonstrate the presence of intracellular viral particles, possibly due to the nondescript shape of the virions. It is also possible that these particles are examples of lysis-deficient viruses (Fane, 2004). Lysis-deficient viruses exist within the host at relatively low densities and do not possess the genes such as holins, requisite for precipitating host lysis. Lysis-deficient viruses simply wait for an environmental stressor that causes death of their host enabling their release.

Several environmental parameters were investigated for their ability to cause *Synechococcus* prophage induction. Rapid temperature and salinity shocks and phosphate stimulation did not trigger cyanophage induction. However, high light stress did lead to cyanophage induction in the isolate examined, which is consistent with the finding of *Synechococcus* prophage induction in the late winter to early spring. In addition, infrequent induction was observed in the summer and in areas with high levels of ambient cyanophage

(McDaniel et al., 2002; McDaniel and Paul, 2005), indicating that cyanophage induction may have already occurred.

These studies indicate that myoviruses are a dominant morphological type among lytic cyanophages. Lytic cyanophages can have variable host range and virulence cannot be readily linked to the type of environment from which a phage was isolated. Experiments with both cultured isolates and natural populations suggest that prophage induction in *Synechococcus* can respond to environmentally relevant cues. In addition, prophage induction appears to be relatively common, at least among cultured *Synechococcus* isolates, and induced cyanophages may be very different from typical lytic cyanophages.

Experimentation with the putative temperate phage from *Synechococcus* isolate P99-14 has been consistent with a ssDNA genome. This is a unique finding because relatively few ssDNA bacteriophages have been described to date. The two families of ssDNA bacteriophages recognized by the International Committee on Taxonomy of Viruses (ICTV) are *Inoviridae* and *Microviridae* (van Regenmortel et al., 2000). The *Inovirus* family is characterized by a filamentous morphology, but members of the *Microvirus* family demonstrate a similar morphology to the induced cyanophage. The type strain and first described *Microvirus* was the coliphage phi X174 (van Regenmortel et al., 2000). Recently described members of the family *Microviridae* are bacteriophages of pathogenic bacterial phyla such as *Bdellovibrio* (Brentlinger et al., 2002), and *Chlamydia* (Liu et al., 2000). Most *Microviridae* are lytic bacteriophages, however, there is genomic evidence that one of the *Chlamydia* phages may be temperate (Read et al., 2000). To the best of our knowledge, if the induced cyanophage proves to be similar to other *Microviridae*, it will be the first described example of a marine ssDNA phage.

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