Phage Communities in Hot Springs

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In extreme environments such as hot springs, phage are the only known microbial predators. We have preformed the first studies of prokaryotic and phage community dynamics in these environments. Phage were abundant in hot springs, reaching concentrations of several million viruses per milliliter. Hot spring phage particles were resistant to shifts to lower temperatures, possibly facilitating DNA transfer out of these extreme environments. The phage were actively produced, with a population turnover time of 1-2 days. Phage-mediated microbial mortality was significant, making phage lysis an important component of hot spring microbial food webs. Together, these results show that phage exert an important influence on microbial community structure and energy flow in extreme thermal environments. In addition to community dynamics, we are now measuring phage diversity in hot springs. To do this, DNA from complete hot spring phage communities has been isolate, cloned, and sequenced. These metagenomic analyses show the these phage are very novel and diverse[1].

At the NAI-Virus Focus Group (NAVIFOG) Workshop/Field Trip we will follow up on the above experiments in 3 ways.

1) Measure survival rates of phage exposed to desiccation and vacuum, as well as temperature and pH changes. For these studies, phage communities will be isolated from both extreme (Little Hot Creek, Mono Lake) and non-extreme environments (e.g., Mammoth Lake). This will provide insight into how well phage might survive the rigors of space travel.

2) Our earlier studies of hot spring phage community dynamics did not address the relative importance of temperate versus lytic behavior. Therefore, we propose to perform filtering and *in situ* incubation experiments to measure the relative importance of these two processes in hot springs (and possibly Mono Lake).

3) Gather DNA and RNA samples for metagenomic analyses. So far, our metagenomic analyses of hot spring phage communities only consists of one time point. Therefore, we will collect nucleic acid samples for comparison purposes.

We will bring tangential flow filtration units with us, as well as supplies and tools for sample collection and preservation.

[1]All of the metagenomic analyses has been done in collaboration with David Mead and Tom Schoenfeld of Lucigen Corporation (Madison).

Great Balls of Fire: Bacteriophage and Microbial Diversity of Boiling Thermal Pools

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Abstract

Boiling thermal pools are unique ecosystems to study microbial ecology, biogeochemistry and evolution, because they are physically isolated from other ecosystems, they are relatively biologically simple, and they are among the most hostile environments known. The water column of boiling hot springs represents an extreme environment that has not been previously explored for viruses or microbes. Planktonic organisms found in the water column potentially originate from significantly higher temperatures and pressures than those found in surface sediments. For example, water temperatures as high as 238°C have been measured in shallow drill holes (< 330 m) in Yellowstone National Park. Bacteriophage and host cells emanating from this formidable environment have important implications for a number of disciplines.

Constructing genomic libraries from extreme environmental niches is challenging due to the low abundance of microbes and bacteriophage; for example, as little as 10,000 cells per ml are found in thermal aquifers, yielding only picogram amounts of DNA. Lucigen has developed methods to make complex gene libraries from anonymous DNA sequences, starting with less than a picogram of purified material. Improved sampling methods and our NanoClone, Single Cell Genomics and CloneSmart technologies have allowed construction of complex community genomic libraries from very limited samples of directly isolated microbial and viral DNA. Limited 16S rRNA sequencing of NanoClone libraries made from Bath hot spring has revealed at least 20 distinct Bacteria and Archaea, many without significant similarity to cultivated microbes. Sequence analysis of approximately 5,000 reads from a bacteriophage metagenomic library derived from Little Hot Creek also shows limited similarity to the NCBI database and a surprising degree of diversity. Comparative data analysis of phage DNA from four different thermal pools will provide a unique glimpse into the diversity and community makeup of a unique set of environments.

The goal of this field trip is to collect additional bacteriophage and microbial samples from new hot springs to construct metagenomic libraries for sequence analysis and expression studies. Analysis of the diversity of bacteriophage genes in different thermal pools from geographically similar and isolated sites will begin to answer important questions about the ecology of hot springs. We will use methods similar to those employed by Breitbart et al. (Genomic analysis of an uncultured marine viral community. Proceedings of the National Academy USA 99:14250-14255, 2002). D Mead will collect samples on this trip and they will be processed at Lucigen for phage and microbial expression analysis (T Schoenfeld, R Godiska, P Brumm). Data analysis will be performed by F Rohwer.

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ABSTRACT
Morphological and biochemical characterization of viral communities in extreme environments.

Viruses are the most diverse and numerous microbes in aquatic systems, but their ecology in extreme environments remains poorly studied compared to oceans and freshwater lakes. Extreme environments are likely to harbor many novel viruses. Although differences in viral assemblages among diverse environments is proximally controlled by the host presence and diversity, lytic viruses must also be adapted to survive as free virions in the chemical and physical conditions of their particular habitat. Comparisons among diverse environments may thus reveal themes in the evolution, adaptation, and diversity of viruses.

We propose to obtain an overview of the characteristics of the free virions within hot springs and /or Mono Lake to seek novel morphologies and relate these to physical properties and biochemical composition. Viruses would be harvested by filtration and ultrafiltration in the field. In the lab the viruses in the concentrate would be subjected to 2-dimensional physical fractionation to determine the range of biophysical and biochemical properties present in the assemblages. The viral concentrates will be fractionated in a CsCl equilibrium density gradient. Each CsCl fraction will then be separated in a second dimension by ion exchange chromatography. Fractions will be examined by transmission electron microscopy to document morphologies and analyzed to determine the associated nucleic acid size and type (RNA vs DNA). Given sufficient starting material, additional analyses could also be performed on the archived viral fractions (biochemical composition, genome sequence, proteome analysis).

Applicants: Grieg F Steward, Asst Professor; Jennifer R. Brum, Graduate Student

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Searching for Crenarchaeal Viruses

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Hypothesis & Goals: We hypothesize that viruses related to novel viruses replication in Crenarchacta hosts in found in Yellowstone's high temperature thermal environments will also be present in the high temperature environments located in the Mammoth Lakes region of eastern California. A comparative analysis of such viruses, we hope, will provide insights into the diversity and evolutionary history of these unusual viruses. We are also interested to determine if related viruses are also present in more neutral/basic represented in the Mammoth Lakes region. The goal of this trip will be to collect appropriate samples for isolation of viruses.

Techniques to be used: Both aerobic and anaerobic field sample from water and soil samples > 80C will be collected from each sampling site. Approximately 50 ml or 50 g samples will be collected from each site. Back in the laboratory, total DNA will extracted from each sample which will be used as templates for PCR-based detection of SSV-, SIRV-, SIFV-, STIV-, and AFV-like viruses. In addition, we will attempt to establish both aerobic and anaerobic enrichment cultures from all field samples. These cultures will be directly screen for virus like particles by transmission electron microscopy. All materials and results will be available to all interested parties.

TITLE: Effect of Mono Lake and Hot Springs Creek phage on bacterial community carbon metabolism.

NAME: Ray Kepner, Marist College

Investigators have demonstrated that Mono Lake contains an abundant & molecularly diverse viral assemblage (*e.g.*, PFGE work of Jiang, Steward *et al.*), while others (Stedman *et al.*) have worked with viruses of thermophilic *Sulfolobus spp.* microbes more typical of acidic hydrothermal systems. Little is known regarding how either lytic, lysogenic, or other types of phage influence ecosystem function in either Mono Lake or nearby thermal waters of the Long Valley caldera. Perhaps phage play a demonstrably important role in regulating the physiological capabilities of the community as a whole.

I propose a simple, preliminary study designed to assess the relative impact of viruses on carbon metabolism by both aerobic Mono Lake bacteria and by other aerobic thermophilic bacteria (*e.g.,* those from Hot Springs Creek). "Natural" bacterial assemblage carbon-source utilization patterns will be compared to those of induced (mitomycin-C) and virus-spiked bacterial assemblages. It is hypothesized that bacterial community diversity, with respect to sources of utilizable carbon, will be reduced upon exposure to both a) higher concentrations of potentially-lytic phage occurring naturally in the water, and b) excised prophage induced by the mito-C treatment.

The proposed fieldwork would involve:

1. Collection of small-volume (1.0 L) water samples with standard membrane filtration to remove most eukaryotic organisms
2. Field concentration and storage of the viral-sized fraction from large-volume (10-100 L) water samples by tangential-flow filtration (30 kDa)
3. Return of concentrates (*i.e.,* retentates) and water samples to Marist College

Back at the lab we would:

4. Enumerate viruses (as virus-like particles) in both small-volume and retentate samples by epifluorescence microscopy following SYBR-Gold staining
5. Induce prophage in small-volume subsamples by addition of 1 μg mL^{-1} mito-C
6. Inoculate 96-well BiOLOG EcoPlates™ with: a) untreated "natural" water samples, b) mito-C induced water samples, and c) water samples spiked with viral concentrate (*i.e*,. TFF retentate) to increase viral density by $\sim$100
7. Incubate inoculated plates at *in situ* temperatures and analyze plates in a microplate reader (590 nm) at defined time intervals
8. Statistical analysis of data would be by PCA and specific changes in utilizable carbon-sources, should they occur, would be noted.

Viral concentrate and other samples, and data collected as part of this exercise will be available to all other NAVIFOG Workshop/Field Trip participants. Results from the proposed work will add to our knowledge regarding the ecological importance of viruses. Wonderful advances have been made in understanding the diversity of phage genotypes. This work would add a tiny bit of "What are they doing?" onto to our increasing knowledge of "Who's there?" with respect to viruses in extreme environments.

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An important goal in microbial ecology is to understand community structure, and the ways in which community dynamics and diversity can differ through time and in space. Environmental heterogeneity should play a key role, due to its proposed influence on the evolution of niche breadth, on the quantity of genetic variance within populations and on patterns of species diversity across landscapes. Although the archaea comprise one of the three major domains of life, relatively little is known of their natural community structure or their phages. In the proposed work, we predict that temporal and spatial changes in the salinity concentrations of solar salterns will strongly impact the profile of resident haloarchaea communities and that variation in haloarchaeal diversity will simultaneously affect halophage diversity due to differing host availability. The pressing need for antibiotic alternatives is great and many advocate a return to studying and developing phage use as therapeutic and prophylactic treatment. Efforts to model the population dynamics of host-phage interactions using computer simulations are worthwhile. But these studies cannot capture the complexities of the population and evolutionary dynamics of host/phage interactions in controlled laboratory environments, much less those occurring in heterogeneous habitats such as within infected hosts. This research proposes to study how environmental heterogeneity affect host-phage dynamics using haloarcahea and halophages as a model.

Abstract of application to participate in NAI-Virus Focus Group (NAVIFOG) Workshop/Field Trip, Mammoth Lakes, California, 22-24 June 2004.

Production of video archive and supporting website.
Emma Hambly

The primary aim of this project is to document the work of the NAI-Virus Focus Group and the workshop/ field trip to Mammoth Lakes as a short (ca. 10 minute) film. The film will be available to be deposited on the NASA Astrobiology Institute website video archive and to all workshop attendees for use in their educational activities. Further, it is hoped that the film, or another edit of the same material, will be commercially transmitted on television. The film will primarily be aimed at a mid to high level undergraduate audience and the broader scientific community, although it is hoped that it will also be comprehensible to interested members of the general public and high school students.

More specifically the film will comprise an introduction to astrovirology through an overview of the work of the members of the NAI–Virus Focus Group. This will include consideration of the scientific questions posed by the NASA Astrobiology Institute and the individual investigators. It will include the aims of studying viruses in extreme environments, the potential roles of viruses in the origin and evolution of life on earth, and discussions of what it is currently possible to address directly, and some of the experimental limitations of these studies.

Interviews with individual investigators will be recorded through the course of the workshop, to fit into a planned film outline. Specific questions that are being addressed will be distributed to participants shortly before the workshop in order to allow some time for their consideration.

The workshop and field trip footage, and possibly some laboratory scenes, will be interlaced within this framework in order to show the reality of sampling and experimentation in this environment. This portion of the film will be relatively unplanned, in the hope of maintaining some of the spontaneity of fieldwork in the final film.

In support of the film still photographs and information assimilated from the NAI-Virus Focus Group members, not only the field trip participants, will be deposited on a website (it would seem appropriate that this would be the NAI-Virus Focus Group website). The film and supporting media will be designed to encourage education and understanding of current issues in astrovirology-related virus research by the public and the wider scientific community, and to enhance interdisciplinary knowledge within the field.

The film and website will be available to go on-line three months after completion of the workshop / field trip. The copyrights of all still photographs, video footage and film imagery, and website design work collected and undertaken in this process will remain the property of Emma Hambly.

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Lateral transfer of photosynthetic genes to and from *Prochlorococcus* viruses

Here we report the presence of genes central to oxygenic photosynthesis in three representatives of two families of double-stranded DNA viruses (*Myoviridae*, *Podoviridae*) known to infect the oceanic cyanobacterium, *Prochlorococcus*. All three viruses (phage) contain genes which encode a core reaction center protein (D1) and a high-light inducible protein (HLIP) that are associated with the photosynthetic membrane. In addition, each myovirus encodes further proteins including a second core reaction center protein (D2) in one and two photosynthetic electron transport proteins (plastocyanin and ferredoxin) in the other. All of the genes encoding these proteins are full-length, conserved and clustered in each genome suggesting a functional role that affords an adaptive advantage during phage infection. We hypothesize that these genes allow host photosynthesis to continue until lysis. Molecular phylogenies of phage-encoded D1, D2 and HLIP protein sequences suggests that these genes are of common ancestry to those from *Prochlorococcus* and were horizontally transferred between host and phage. Interestingly, phylogenetic inference suggests the D1 and D2 proteins were transferred multiple times from host to phage, while phylogenetic and clustering analyses with the highly divergent HLIP multigene family suggests that these genes have been acquired multiple times from their host cells, but after a period of evolution in the phage have been laterally transferred back from phage to host. In addition to the proposed functional role for these phage-encoded functional genes, we hypothesize that through lateral gene transfer these *Prochlorococcus* phage are also driving the niche differentiation of their hosts.

Voigt Abstract

Therapeutic bacteria have the potential to treat a wide range of disease. Their intrinsic ability to preferentially target and replicate within tumor masses has been exploited to develop a *Salmonella* strain as an intravenous anti-cancer therapy, which is currently in phase I clinical trials (Toso *et al.*, *J. Clin. Oncology*, 2002). Existing bacterial therapeutics rely on using a pathogenic strain where the virulence is attenuated via gene knockouts. In this talk, I will describe our efforts to synthetically design a non-pathogenic strain of *E. coli* to specifically target and destroy cancerous cells. This requires the engineering of a bacterial touch sensor that can distinguish between healthy and cancerous cells, the introduction of virulence systems such as the Type III secretion apparatus, and the introduction of robust regulation to wire it all together. To facilitate this process, we are also developing computational models of the regulatory network controlling *Salmonella* pathogenesis. In addition, I will describe bioinformatic tools to predict which circuits and components out of the growing database of sequenced bacterial genomes are likely to function robustly when transferred into a new host background. These techniques will broadly aid the forward design of bacteria for applications in synthetic biology.

A natural extension of this work is to study viruses, both as the primary vector of the therapeutic as well as a means to identify novel circuits and components that will function robustly in a bacterial host background. In particular, bacteriophage often contain circuitry that can be introduced and programmed in a broad range of hosts. They can also be vectors promoting the hyperevolution of genes that produce a benefit to the bacteria and may be involved in gene transfer between different trees of life. For example, Salmonella contains many lysogenic phage that encode secreted virulence factors, including a modified human gene that perturbs a signaling relay involved in apoptosis.