Phages in nature

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Bacteriophages or phages are the most abundant organisms in the biosphere and they are a ubiquitous feature of prokaryotic existence. A bacteriophage is a virus which infects a bacterium. Archaea are also infected by viruses, whether these should be referred to as ‘phages’ is debatable, but they are included as such in the scope of this article. Phages have been of interest to scientists as tools to understand fundamental molecular biology, as vectors of horizontal gene transfer and drivers of bacterial evolution, as sources of diagnostic and genetic tools and as novel therapeutic agents. Unraveling the biology of phages and their relationship with their hosts is key to understanding microbial systems and their exploitation. In this article we describe the roles of phages in different host systems and show how modeling, microscopy, isolation, genomic and metagenomic based approaches have come together to provide unparalleled insights into these small but vital constituents of the microbial world.

Introduction

We live in a microbial driven world that only exists because Bacteria and Archaea tempered the previously hostile environment on early Earth to create atmospheric conditions that allow eukaryotic life forms to flourish. Bacterial and archaeal encoded enzymes catalyze all the major processes involved in global biogeochemical cycling, playing key roles in the carbon and nitrogen cycles, and producing approximately half of the oxygen in the Earth’s atmosphere. In macro-organisms (animals) prokaryotic cells generally outnumber eukaryotic cells, where they assist in important aspects of survival such as nutrition and defense. So what roles are phages playing in this microbial mix? Once ignored, it is now becoming increasingly accepted that phages play key roles in the biology of microbes, which themselves impact environments at large. Many previous excellent reviews have highlighted the importance of bacteriophages in specific environments for example. In this article we present three case studies to illustrate how an appreciation of the roles of the viruses is pertinent to understanding microbial physiology, population dynamics and evolution. We show how our microbial driven world is tempered by bacteriophages. To contextualize the case studies we summarize the history of phage research and give an introduction to the biology of bacteriophages. We review their distribution and describe how they are enumerated and characterized. Finally we discuss the ways in which phages may influence their host’s evolution and population dynamics.

Brief history of bacteriophage discovery and research. Bacteriophages were first discovered in 1915 by William Twort, and in 1917 by Felix d’Herelle realized that they had the potential to kill bacteria. After a pre-antibiotic era heyday they were then essentially disregarded as significant therapeutic agents in the West, primarily due to the comparative ease by which antibiotics could be administered. Research and the practice of using bacteriophages did continue in some countries such as Georgia (as part of the former USSR), where they were, and continue to be routinely isolated and used to treat a large number of diseases. Bacteriophage research then focused on a number of model phages which primarily infected E. coli. These studies provided the back-bone of modern molecular biology, for example phages were used to identify the basis of genetic material, and that 3 nucleotides code for an amino acid. They also allowed the identification of restriction enzymes. For several decades, only a handful of phages were studied in great detail. The recent renaissance seen in phage biology has been triggered due to a growing awareness of the number of phages in all bacterial dominated environments (as revealed by epiflourescent and electron microscopy, and from molecular studies), and indeed in the genomes of bacteria following whole genome sequencing projects. This checkered history has resulted in a patchy knowledge of phage biology but with enough observations for scientists to realize that phages are dictating many aspects of Bacterial/Archaeal biology. These observations have invigorated an invigorated interested in bacteriophages, and are part of the stimulation for this journal Bacteriophage, in which this article is written to illustrate the roles that bacteriophages play in the natural world.

Phage life cycles. In order to appreciate the roles of phages in nature, an understanding of their possible interactions with their hosts is necessary. Phages have various possible life cycles which, along with interaction with their physical environment, dictate their role in bacterial/archaeal biology. The lytic life cycle is where phages infect and rapidly kill their infected host cells, thereby shaping bacterial population dynamics and occasionally assisting in their long term evolution via generalized transduction. The lysogenic life cycle in contrast, is where phages instead of directly killing their hosts, integrate into their host genome, or exist as plasmids within their host cell. This lysogenic life cycle can be stable for thousands of generations and the bacteriophage may
alter the phenotype of the bacterium by expressing genes that are not expressed in the usual course of infection in a process known as lysogenic conversion. A well known example of this is the gene associated with *Vibrio cholerae* which encodes the toxins that cause cholera symptoms.11 Phages may also have a pseudolysogenic component to their life cycle. This is a controversial concept, and has many different definitions within phage biology.3 We define it here as the situation that occurs when a phage has entered a bacterial cell and doesn’t integrate in a stable fashion, but will stay in this ‘mode’ until conditions occur which trigger them to enter into the lytic or lysogenic life cycle.7,12 We illustrate how pseudolysogenicity is difficult to study, yet may be important in markedly different systems. Finally there is the chronic infection lifestyle found in some archaeal viruses, in filamentous phages (rod shaped single stranded DNA phages), and in plasmaviruses which infect *Mycoplasma*. In this life cycle phages are slowly shed from the cell over a long time period without obvious cell death.

**Phage abundance and diversity.** Having considered the possible phage life cycles, it is logical to review where phages are found, and how they are enumerated and characterized. Specific examples of phages and their characterization will then be given in the case studies. The first approaches that led to the realization of phage abundance were based on epifluorescent microscopy following DNA staining which suggested that in sea water there are around 10 phages in existence for each bacterial/archaeal cell.7,13,14 Similar figures have been shown for freshwater environments, but for other more complex environments the situation is less clear and virus numbers may either be higher or lower than that of their bacterial/archaeal hosts.15

As phages have an obligate requirement for a host, their abundance and distribution is likely to be based on that of their host organisms. Therefore to make sense of viral abundance, one must establish where the majority of their hosts exist. Although we are often focused on bacterial pathogens, most of the Earth’s Bacteria and Archaea are found in the open ocean, the soil and in ocean sediments, and terrestrial sub-surfaces where there are an estimated 1.2 x 10^29, 2.6 x 10^29, 3.5 x 10^30 and 0.25–2.5 x 10^30 cells respectively.16 Bacteria and Archaea are often associated with humans and animals which provide many niche environments within them, often where these micro-organisms have become an essential symbiont. Despite animal abundance on the Earth the total number of prokaryotes associated with them is several orders of magnitude less than for the major land and ocean environments. For example, in humans the majority of prokaryotes are found in the colon, so multiplying the total human population of 6.8 x 10^9 by the number of prokaryotes per gram of human colonic matter (3.2 x 10^10), by the average amount of colonic material per human of 220 grams, gives an estimated total of 4.8 x 10^33 prokaryotes (based on ref. 14, and on the UN 2009 current estimate of the human population size). Although not as numerically significant, bacteria are of essential importance when associated with humans, particularly either in a disease, or a food producing context when bacteria are associated with causing disease, or where we are reliant on bacteria, for example for cheese production, which can fall prey to bacteriophage attack. Therefore, in terms of human impact, an appreciation of the roles of bacteriophages which infect these bacteria is of paramount importance.

**Traditional approaches to the quantification and characterization of bacteriophages.** There is no single method that can be used to establish how many phages in an individual sample can infect a specific host however traditional and molecular approaches can be usefully combined to build up a picture of the viral community. The number of phages which infect all hosts can be determined using epifluorescent microscopy, or flow cytometry27,16 and the morphological diversity of phages using transmission electron microscopy (TEM).19 Currently the number of phages which infect specific hosts can only be determined from isolation approaches.20 For isolation studies, suitable hosts can either be isolated specifically from the environment of interest, or a model permissive host can be used. Clearly these approaches only identify phages that infect the specific strains being used as a host and so it is difficult to establish what proportion of phages present are being isolated. Phages may be present which infect the species being used in isolation, but may not infect the model strain if the strain lacks the appropriate phage receptors, has a restriction system, if abortive infection occurs, or if it has a CRISPR (clustered regularly interspaced short palindromic repeats) defense system.21-23 Furthermore, phages isolated are amenable to propagation, and not necessarily representative of the most abundant phages in natural populations. It is hoped that future work based on single cell sequencing will provide additional data on the nonculturable viruses that are important in natural populations.

**Molecular approaches to the quantification and characterization of bacteriophages.** There is no universal marker for phages in the same way the 16S rRNA gene can be used to reliably place the phylogenetic affinity of all bacteria. This is because there are no genes that are suitably conserved within all phages, or even for example present within one taxonomic group such as the bacterial virus order Caudovirales.24 However, there are several examples of smaller taxonomic group specific markers, which are extremely useful for assessing phage diversity and abundance. For example, researchers commonly target genes, which encode structural proteins as phylogenetic markers. One gene which has been widely used is the gene which encodes the portal protein which is located at the top of the neck of the phage and through which DNA passes en route down the tail sheath.25-28 The same primer sets have been used to investigate these sequences in T4-type phages which are known to infect a wide range of bacterial hosts.29 As well as providing estimates of diversity, molecular markers can also offer new ways of quantifying bacteriophage abundance which are free from the isolation based complications discussed in the paragraph above. For example molecular markers based on the Q gene and a gene encoding for a shiga toxin, revealed a far greater abundance of shiga toxin phages present in the soil than was observed using standard isolation based approaches.30

Other molecular approaches to assessing bacteriophage diversity are where markers based on restriction fragment length polymorphisms (RFLP), or on denaturing gradient gel electrophoresis (DGGE) are used to assess the diversity of a bacteriophage.
Metagenomics. The newest way of assessing phage diversity and indirectly abundance is using viral metagenomics. This is where the total viral component from a particular environment is collected and sequenced. This approach has been made possible due to the progress in sequencing technology, and the reduction in cost which has made it relatively affordable. Protocols vary according to the sample in which the phages are present but bacteria are always removed, and often where the total amount of viral DNA is low, enrichment steps are carried out to amplify the total viral community DNA so there is enough to sequence. 31-33 Metagenomics can be used to identify phages or phage genes of environmental significance, such as those that are highly abundant or specific to particular niches. 34-36 This allows data to be collected on the dominant viral genomes present in a specific location, without having to culture their hosts and isolate phages, and provides a great starting point for understanding the roles that bacteriophages may be playing. It can also provide information on phages that are not amenable to propagation, or that do not have hosts in culture. It is estimated that 95% of bacteria cannot be cultivated under laboratory conditions, so consequently the phages which infect them cannot currently be isolated either. 37-38 Metagenomics can also potentially provide abundance information based on the amount of coverage of particular phages/gene sets present in sample sets. Clearly there may be amplification, or sequencing bias but over, or under representation of particular genes can yield useful information about phage biology.

The main current drawback to metagenomic studies is that because viral genome diversity is so high, a large proportion of predicted genes are ‘unknown’ or ‘hypothetical’, and therefore currently much of the information collected by this approach is not immediately useful. This situation will improve as additional genomes from isolated bacteriophages are sequenced and annotated, and as bioinformatic tools based on structural protein homologies are developed to assist amino acid or nucleotide sequence comparisons. Finally, it is often difficult to test hypotheses made from sequence data. Although genes of interest from metagenomes can be cloned, expressed and biochemically characterized, their relevance to specific phages can only be established if cultured phages with those genes are in existence.

The big picture: phage abundance and diversity studies. While each enumeration/identification technique contributes one piece of information to our understanding of phage abundance and diversity, few studies attempt all methods in combination. Fewer still collect the necessary metadata needed to establish phage number and hosts identitiy. In other words most studies either count total phage number, or identify a sub-set of phages which are associated with one host bacterial strain. However, each approach has merit, and the body of research based on them is gradually improving our understanding of the phage world. It is really an exciting time to be a phage biologist because very few environments have been well characterized and those that have, have revealed endless surprises in terms of gene content. Therefore, it is likely that significant surprises and unexpected findings will abound as new systems are studied.

An example of the synergy of different approaches can be seen in a recent study which compared cyanophage genomes present in the large scale metagenomes present in the ocean derived CAMERA (Community Cyberinfrastructure for Advanced Microbial Ecology Research & Analysis) data set to those found in culture. Rather pleasingly, the data in the metagenomes reflects the gene content and diversity of the cyanophages that are in culture. 39 Some studies have used multiple methods to characterize viral assemblages; for example a recent study used EM, flow cytometry and metagenomics to characterize the viral community associated with Antarctic lakes throughout an annual cycle. These combined approaches revealed many interesting features of the virus communities in Arctic systems such as the shift from a ssDNA virus dominated community in spring when lakes are generally iced over, to a dsDNA dominated community in the summer. 40

Bacteriophage biogeography and persistence. Recent evidence suggests that prokaryotes may exhibit biogeography i.e. be endemic to particular environments which goes against the idea that “everything is everywhere.” 41,42 This idea leads to the possibility that phages can also show biogeography. Studies have shown that some phage have a global distribution while others may be endemic to particular environments. A recent review on this subject reports that a 2009 meeting of the Scientific Committee on Ocean Research Viral ecology Working Group concluded that this question remained unanswered. 43 In this review we consider this notion more under our case studies.

Generally phages are pretty stable if the environment is not hostile. They are broken down in UV light, and can be damaged by abrasion, or exposure to chemicals, but researchers have been known to keep phages in their fridges for over 40 years with no reduction in titre (Ackerman HW, personal communication). Unpublished work by Suttle, and by Clokie has demonstrated that cyanophages can be isolated from sediments that are several decades old (Clokie MR, unpublished). Finally some bacteriophages seem remarkably unstable in the laboratory and Clokie et al. have observed that both Clostridium difficile bacteriophages, and those which infect Streptococcus pneumoniae reduce in titre on a weekly basis regardless of the buffer/media they are stored in (Clokie MRJ et al., unpublished).

Impact of phages on host populations. Having defined the possible life cycles and ascertained what is known about phage diversity and abundance, it is pertinent to review what is known about how bacteriophages impact their host populations. Several approaches have been used to determine the impact phages have on their host’s populations. Experimental evidence from chemostats and observations of phages/hosts in open systems have shown that for some bacterial species, populations of phages and hosts oscillate with time. 44,45 The relationship between phages and their hosts has been modeled, and in a simple environment if there is no cost to host resistance the same oscillation in populations occurs. 46 However, if there is a cost to phage resistance, then bacteriophages have been theoretically and experimentally shown to drive host diversification. 47-49 Diversification of bacteria
may occur in the phage receptor region, which may be related to nutrient uptake, or it may occur possibly on a faster timescale on CRISPR elements that can quickly evolve to provide a host defense system. An alternative dynamic between hosts and phages exists where phages have a temperate life cycle whereby phages may contribute to the success of their host bacteria by encoding useful genes; examples of this dynamic have been hypothesized following many bacterial genome sequencing projects, and through recent metagenomic studies.

To illustrate the major roles that phages play in microbial ecology, physiology and evolution we describe in detail case studies from three contrasting systems of phages in their natural settings. The first case study describes marine cyanophages because they have been the most extensively studied phages in the marine environment, and, at a broader level, they probably constitute the group of phages that has been studied from the greatest number of perspectives and therefore significant data exists to begin to unravel their roles in cyanobacterial biology. They have been isolated from around the world, have been the attention of 10s of genome sequencing projects, and much metagenomic sequencing effort, their impact on host dynamics has been studied in natural systems and expression work and modeling have begun to establish the biological significance of particular features of their genomes. The second case study reviews our knowledge of the roles that phages play in animal bodies. Despite this environment being closer to home, the complex nature of the environments, and a surprising paucity of research effort mean that it is less well understood. We predominantly focus on phages which infect gut E. coli (coliphages) as they are abundant in animal bodies and enough significant data exist on them for an understanding of their relevance to be postulated. Finally we discuss the ‘phages’ which infect the Archaea domain as an example of how little is known about this intriguing group of organisms. Within each case study we briefly summarize the history of research in the field and describe how the phages have been isolated and characterized. We describe the impact of molecular and metagenomic studies on the fields with a view in all three systems to ascertaining the impact of phage on their bacterial/archaeal hosts, how they shape and control their host populations. In all systems, we also highlight the areas where conflicting data, or lack of it means that the role of phages has yet to be established. We hope that the concepts brought out in this review will form an appropriate framework which will be helpful when considering novel, or less studied groups of phages, or when considering the exploitation of phages.

**Case Study: Marine Cyanophages**

Cyanophages: Discovery and distribution. Although several heterotrophic phages have been studied, this review focuses only on the cyanophages for which most data is available for. Their study in the marine environment began in the early 1980’s when phages infecting both unicellular and filamentous cyanobacteria were observed in the Black Sea. In the early 1990s, research into began in earnest with the isolation and characterization of phages infecting marine Synechococcus. Ten years later cyanophages that infect the closely related Prochlorococcus were also isolated. These two genera of cyanobacteria are the predominant primary producers of the nutrient poor (oligotrophic) areas of the ocean which cover around 70% of the surface of Earth. Remarkably, these two genera Synechococcus and Prochlorococcus account for up to 50% of primary production the world’s oceans, Prochlorococcus is generally found in the warmer oceanic waters between the 40° latitude north and 40° latitude south whereas Synechococcus is much harder and is found on either side of those latitudes. Therefore the study of the viruses which infect these organisms has significant ecological interest in terms of global carbon cycling.

**Isolation and characterization.** The early work on cyanophages concentrated on determining their abundance in the environment using isolation based approaches, and then characterising the isolated phages using well or plate assays to determine host range and burst size. These studies revealed that cyanophages are widespread in the environment, at concentrations as high as 1 x 10⁶ pfu ml⁻¹. They all had relatively long latent periods of several hours and their burst sizes range from ~20 for the cyanophage S-PM2 to ~250 for cyanophage S-BBP1. TEM analysis has revealed a large diversity of cyanophage morphotypes, with phages observed from the Myoviridae, Podoviridae and Siphoviridae. Most cyanophages that have been isolated to date however belong to the Myoviridae, and are morphologically similar to T4-like phages.

**Molecular approaches to cyanophage study.** An important milestone in the study of cyanophages was the development of molecular tools to study their distribution and diversity. The discovery by Fuller et al. that cyanophages have a gene that is homologous to g20 in T4, was surprising at the time, and allowed the development of a set of PCR primers to specifically amplify this gene from cyanophages. Several studies using this initial primer set, or improvements on it, to target g20 from a wide range of geographical locations, and ocean habitats. A similar approach was taken to target the DNA polymerase gene of cyanophages from the Podoviridae family. Both of these studies have revealed how widespread cyanophages are within the oceans, and have revealed a clear lack of cyanophage biogeography based on either gene.

**Surprising synteny and homology between cyanophages and enteric viruses.** The work of Hambly et al. extended the work above by sequencing the genes adjacent to g20 and to show that the cyanophage S-PM2 shares a conserved module of genes with the model E. coli phage T4. The subsequent sequencing of cyanomyovirus genomes has revealed that there is an even greater amount of synteny and homology between them and T4. Although this may seem like a diversion from the main thread of examining bacteriophage roles in nature, it suggests that bacteriophages do share a commonality in terms of the way in which they function in many bacterial groups. Figure 1 shows a dendrogram describing the relationship between cyanophages and other myoviruses. This is based on the presence or absence of specific genes. Although it clearly demonstrates that cyanophages are more closely related to each other than to other T4-like phages, they do share some genes with the T4-like phages KVP40, RB49
and T4. Podoviruses which infect cyanobacteria are also related to enteric phages with the genomes of cyanophages P60 and P-SSP7 being T7-like in terms of architecture and gene content. However, this appears not to be the same in the siphoviruses. For example the genome of the siphovirus P-SS7 that infects Prochlorococcus is very distinct from other lambdoid like phages. This is the only Prochlorococcus siphovirus reported so far, others may of course be different and share similarity with other known siphoviruses.

**Cyanophage genomes.** The availability of sequenced genomes has allowed detailed genome comparison studies of cyanophages. Twenty-one marine cyanophages have been sequenced to date; fifteen myoviruses, five podoviruses and a siphovirus. This number is expected to rise rapidly in the next few years; for example one initiative being funded by the Gordon and Betty Moore Foundation is sequencing dozens more cyanophage genomes and viromes (all viruses in a habitat). This is likely to provide major insights into the biology of these organisms, and to highlight further the roles phages are playing in host biology.

**Impact of cyanophages on the physiology of their hosts during infection.** Clues to how cyanophages influence the biology of their hosts have been obtained from gene expression studies of infected model systems. Research has shown that while cyanomyoviruses share many genes with T4-like phages, they do not have the same expression patterns. T4 has a beautifully choreographed expression pattern with genes transcribed with early, middle and late profiles. Cyanophages lack a “middle” mode of transcription pattern, and consistent with this they lack the middle promoter activational genes MotA and coactivator AsiA which control the middle expression genes in T4. The cyanopodoviruses also encode and express a number of genes that are homologues to host genes. The Prochlorococcus cyanophage P-SSP7 has been demonstrated to increase the expression of a number of host-encoded genes during infection. This observation is thought to result from both the host stress response to infection, and to activation by phage encoded factors.

‘Host’ encoded phage genes illustrate the intertwined relationship between phage and host. A number of genes have been found in cyanophages that are homologous to genes found in their cyanobacterial hosts. These genes are often referred to as “host genes” or more recently AMGs (auxiliary metabolic genes). Among the most interesting AMGs identified in cyanophages are the genes involved in photosynthesis which has led to some cyanophages being referred to as "photosynthetic phages". S-PM2 was the first cyanophage discovered to carry the essential photosynthesis genes psbA and psbD which encode the D1 and D2 proteins respectively. The D1 and D2 proteins form a heterodimer at the core of photosystem II. An unavoidable consequence of oxygenic photosynthesis is the release of reactive oxygen species, that can damage the PSII complex, specifically the D1 polypeptide. As a consequence of this, a repair mechanism has evolved in all oxygenic phototrophs, which removes and replaces the damaged D1 protein. It is postulated that the expression of phage encoded D1 protein ensures a source of energy for phage replication, by maintaining photosynthesis after host protein synthesis has ceased to be expressed. This is supported by the fact that both phage encoded psbA transcripts and the corresponding D1 polypeptides are increased during the infection cycle. Furthermore recent modeling studies using in silico deletions of psbA from P-SSP7 predicts that under high-light conditions there is an increase in burst size for phages carrying psbA.

Genome sequencing, PCR screening and comparative genome hybridization have revealed that psbA and psbD are widely distributed in cyanophage isolates and the environment at large. Although psbA and psbD are widespread, they are not found in all cyanophages, and only psbA is thought to be part of the “core-genome” of cyanomycoviruses. The siphovirus P-SS2 lacks both psbA and psbD; at present it is not known if this is common feature of siphoviruses.

Cyanophage genomes contain a many other ‘bacterial’ genes that might maintain or alter host physiology. Many of these genes are thought to have been acquired as a response to the harsh environment that cyanophages inhabit; the high-light oligotrophic open ocean. Other genes linked to high light conditions include the high-light inducible (bli) genes, a PTOX-encoding gene postulated to provide an alternative mechanism to reduce photo-damage, electron transport protein encoding genes, and genes encoding proteins required for synthesis of the light-harvesting phycobilisomes complex. Additionally several genes that encode enzymes found in the pentose-phosphate pathway have been found to be common in cyanophages and are thought to allow optimization of necessary NADH and ATP for phage replication. A phosphate transport encoding gene found only in cyanophages isolated from phosphorous limited waters provides further evidence that the environment shapes cyanophage genomes to have several features that may benefit them.

Metagenomic studies have extended observations of ‘bacterial’ genes in phage genomes, and they have shown the vast extent to which the cyanobacteria and phages have evolved together.
Life cycles and marine cyanophages. P-SS2 is also the first marine cyanophage that has been isolated and had its genome sequenced that is thought to be temperate. At present the overwhelming majority of cyanophages that have been isolated are obligately lytic. This is somewhat surprising as there are numerous reports of lysogeny occurring in the environment in cyanobacteria. However, despite the sequencing of over 20 Synechococcus and Prochlorococcus genomes, no complete prophages have been identified. Interestingly, McDaniel et al. have reported the isolation of a putative temperate phage induced from Synechococcus, however it has a ssDNA genome which is unlike all other cyanophage isolates. The bioinformatic analysis of the P-SS2 genome sequence suggests that it capable of integration into a host genome, although this has not been experimentally demonstrated.

Temperate cyanophages are either rare, or just inherently so unstable to work with that we are just not able to isolate them well yet, and therefore we do not appreciate their roles in cyanobacteria. The lack of isolated cyanobacterial lysogens means that nothing is known on how cyanophages may change host physiology via lysogenic conversion. Consistent with no isolated cyanobacterial lysogens, no prophages have been observed in cyanobacterial genomes as there are for heterotrophic bacteria.

The pseudolysogenic life cycle may be ecologically relevant in cyanophages. It has been shown that when cyanobacteria are grown in phosphate depleted media (as they would often be growing in the open oceans), then cyanophages enter the cells but do not enter the lytic cycle. Although the genetic nature of this interaction has not been characterized, the observation is consistent with Abedon’s definition of pseudolysogeny being a carrier state where ‘lytic’ phages can remain inside bacterial cells until conditions are suitable to enter most probably the lytic infection cycle. Unpublished data has shown that in this pseudolysogenic state AMG’s are expressed at a higher level than structural phage genes (Clokie MR et al., unpublished).

Influence of cyanophages on cyanobacterial populations. A study over an annual cycle in the Red Sea showed that cyanophages co-vary in abundance and genetic diversity with that of Synechococcus, which is consistent with the hypothesis that cyanophages are an important factor in controlling cyanobacterial secondary ecological succession. The diversity of phages was estimated using DGGE following DNA extraction from the viral and host fraction of the water samples. Similar studies have indicated that cyanophages are involved in structuring the population dynamics of their host community, and driving biogeochemical cycling. Also consistent these observations is a laboratory based study that has showed that when Synechococcus develop resistance to cyanophage infection, they appear to have reduced growth rates. Therefore in a natural setting, infection from phages is a necessary ‘risk’ to achieve suitable growth rates to outcompete the ‘phage resistant’ strains. The ‘cost of resistance’ may also manifest itself in other forms. A recent study found that a spontaneous phage-resistance mutant of Synechococcus was more susceptible to grazing by heterotrophic nanoflagellates. Evidence of cyanophages shaping the populations of hosts has also been observed at a genetic level, with evidence of intragenic recombination of pshA genes between phages and hosts and the horizontal transfer of hli genes to and from cyanophages and Prochlorococcus.

Cyanophage spatial and temporal dynamics. Cyanophage abundance and diversity has been studied at a number of scales from short diel cycles to annual cycles. Generally cyanophage numbers are at their highest when their hosts are most abundant, which coincides with the warmer summer months. As one might expect, cyanophage abundance also varies through the water column, with cyanophage numbers generally found to decrease with depth. The diversity of cyanophages has also been observed to change over temporal and spatial scales with particular genotypes observed only at certain periods of the year or particular depths.

Cyanophage future perspectives and summary. Although we are beginning to understand the importance of cyanophage interactions with their hosts, there are many unknowns to be unraveled. For example no cyanophage tail fibers, or indeed their corresponding receptors have been experimentally demonstrated. A further example of a molecular mechanism used by cyanophages to manipulate and control their hosts was demonstrated with the discovery of the expression of an antisense RNA by the cyanophages S-PM2. While this is the first antisense RNA found in a lytic phage, it is unlikely to be the only one that exists. Antisense RNAs form part of large group of RNAs commonly referred to as non coding RNA that are becoming increasingly found in bacterial genomes, where they act as regulators, adjusting physiology in response to changes in the environment. Therefore, it is likely the antisense RNA in the cyanophage S-PM2 (and other viruses) is also important as part of a response to environmental change.

In summary, estimates of cyanophage infection rates are high with an estimated 50% of all cyanobacteria being infected at any one time. Whether or not they have a defined biogeography is uncertain. Cyanophages were initially thought to be important in terms of diverting the flow of carbon that is fixed by cyanobacteria into the microbial loop, i.e. by infection and the subsequent release of carbon by cell lysis. However, the discovery that cyanophages carry and express photosynthetic genes, suggests that they may also be directly responsible for a significant proportion of carbon fixation in the oceans. Most known cyanophages are essentially lytic and they have been shown to drive host diversity over monthly timescales. Cultured cyanophages appear to be consistent with metagenomic data, and at least 50% of the genes within their genomes are unique. The development of a genetic system will allow the significance of the ‘host’ encoded genes to be understood, and also allow the function of novel genes to be elucidated.

Case Study 2: The Animal Environment

Animal-associated phages: Discovery and distribution. The animal or human organism is a complex microcosm of multiple interconnected ecological systems, many of which are densely populated by microorganisms. An indication of the extent of the microbial biomass can be seen from the fact that it contributes up
to 54% of the total weight of human feces.\textsuperscript{117} Significant numbers of individual species of bacteria are found in the intestines, the oral cavity, the vagina, the respiratory tract and the skin where the number of bacteria present frequently overwhelmingly exceeds the expected threshold levels required for efficient phage multiplication. Where this is the case, a high impact of phage infection on the dynamics of the bacterial populations can be expected.\textsuperscript{118}

Although Felix D’Herelle first noticed that bacterial viruses are a normal part of the microbiota of healthy animals and humans,\textsuperscript{119} our understanding of the role of bacteriophages in shaping and maintaining human symbiotic micro-flora is scarce and fragmentary. The available literature on this subject was extensively analyzed in a recent review.\textsuperscript{120} Here we review the key roles that phages play in the ecology of the symbiotic micro-flora found in animals.

**Isolation and characterization.** The high abundance of phage-like particles in the intestinal microbial systems was first demonstrated by electron microscopy observations in the 1960s\textsuperscript{120-123} these particles represented a vast majority of all virus-like particles (VLP) observed in these kind of samples collected from healthy humans or animals. Despite the early start of the studied of animal and human non-cultured intestinal viral communities, the precise measurements of VLPs concentrations in intestinal contents or feces of any animal were never published. Based on the yield of the total virus (phage) DNA reported in recent metagenomic studies\textsuperscript{124,35} the concentration of VLPs in feces on humans can be estimated as $10^{10}$ ml\textsuperscript{-1} and in horses up to $10^{11}$ ml\textsuperscript{-1}.

**Molecular approaches to animal phage studies.** The diversity of non–cultured intestinal phages was initially characterized by their morphology observed by TEM. This was followed by studies based on the purification of viral communities and analysis of their nucleic acids, initially by pulse-field gel electrophoresis separation to determine genome size, and more recently by metagenomic approaches (reviewed in ref. 120; see also ref. 35). It appears that the vast majority of the intestinal virus-like particles are related to the tailed phages (except for RNA viruses that are mostly plant viruses ingested with food). The estimation of phage diversity based on these data is in the order of hundreds to thousands of distinct genotypes present in one sample of rumen content or feces. In a recent study, as many as 69 morphologically distinguishable bacteriophage types were detected in one specimen of horse feces in over total 200 particles which were examined (Fig. 2).\textsuperscript{126} These results agree well with the data of the metagenomic analyses on horse feces which have shown that even the most abundant phage type only constitutes between 5–10% of the total population.

**Animal phage spatial and temporal dynamics.** Although many bacteriophages and phage-host systems have been isolated and characterized from animal-related sources using culture based approaches, the vast majority of these studies were designed to use phages as biological indicators of water fecal contamination, or for other practical applications. The presence and variation of the phage populations in time and space have only been analyzed in a limited number of studies.

A recently published metagenomic study of viromes of the feces obtained from monozygotic twins and their mothers indicated that their viral communities are stable in time, but differ markedly between individuals within one family, despite the fact that the individual bacterial communities of the close relatives were quite similar.\textsuperscript{33} This is consistent with metagenomic bacterial data.\textsuperscript{125} Consistent with human intestinal data, the composition of the non-cultured viral community in rumenal or intestinal microbial systems also seems to vary significantly between subjects. One major difference however is that unlike the situation in the human intestinal virome, the rumenal virome changes significantly with time (reviewed in ref. 120). In conclusion, unlike our appreciation of bacteria and their niches, there are not sufficient data to attribute some types of phages to “normally” associated with particular ecological processes, or with particular animal species.

**Interaction between phages and hosts in their physical environment.** The environments created by the animal-host physiology, and by the microbial activity in different densely populated niches appears to profoundly influence the mode of interaction between bacterial and phage populations. The intestinal coliphage ecology is one of the best studied examples of this complex interaction and the impact of the phages on host populations, mechanisms of phage-host mutual regulation and adaptation have been shown to vary considerably in different animal species. For example, the environmental conditions found in the mouse gut are not favorable for the replication of E. coli phages for some reason, and no studies have shown that mice excrete any natural coliphages.\textsuperscript{127,128} Furthermore, the resident mouse E. coli populations are almost completely resistant in vivo to externally administrated cocktails of T4 related bacteriophages that kill up to 100% of the same strains in vitro. In contrast to the mouse gut, recent work by Golomidova et al. has confirmed a previously published observation that in a natural situation, healthy horses frequently do excrete coliphages. This work also showed that coliphage populations exhibit significant temporal variation, with up to 4 orders of magnitude difference in phage abundance during 15 days of monitoring.\textsuperscript{129} In terms of coliphages in other animals, a low fecal coliphage prevalence has been reported in dogs.\textsuperscript{130} The role that phages play in the ecology of bacteria, also differs between specific ecosystems within different body sites in the same animal species. The failure to obtain phage isolates from the vaginal and oral cavity in humans suggests that the phage impact in these systems is less than their impact in other environments, such as colonic ecosystems of the same species (see here below and the refs. in ref. 120).\textsuperscript{131-133} However direct electron microscopy observations of some samples obtained from dental plaque material showed that high numbers of VLPs were observed.\textsuperscript{134} Therefore the problem of the phage activity in the oral cavity has to be further addressed, and may be a reflection of our inability to isolate these viruses. A further complication to our understanding of phages associated with animals is the observation that despite relatively high concentrations (about $10^7$–$10^9$ ml\textsuperscript{-1}) of phage particles in the rumenal contents of ruminants, the viruses appear to be unable to control the density of the host populations.
This observation could be due to the physical and chemical impact of these environments, for example the tannic acid and other chemical compounds in the rumen of sheep and cattle appears to significantly inhibit bacteriophage infection of \textit{E. coli}. 

Generally, it seems that the phage ecology in animal-associated microbial systems should be considered as a tripartite interplay between the bacteriophage, the host bacterium and the environment within the macro-organism. In this play, the macro-organism influences both the bacteria and the phages. A direct influence on phages can be seen from their destruction by digestive enzymes and macrophages, their translocation by blood and their transmission between organisms that is facilitated, or restricted due to specific behaviour. Finally, the efficacy of phage infection can be markedly influenced by compounds secreted by the macro-organism such as bile salts (reviewed in ref. 120). This interplay is genuinely three way, and there is evidence that the macro-organism is directly, and indirectly influenced by both phages and microbes. An example of phage influence on the macro-organism can be seen from recent data which suggests that phage particles may interact directly with immune competent cells, exerting immuno-modulatory activity. 

Life cycles and animal bacteriophages. The identification of a number of coliphages isolated on laboratory \textit{E. coli} strains and on the field isolates of horse fecal coliform bacteria from the same samples, suggests that the vast majority of the naturally occurring coliphages of the horse gut are lytic. This observation contradicts the observations that Furuse and colleagues made on human associated coliphages. These authors found that in feces of healthy subjects, the coliphages are present at fairly low titers, and are mostly temperate. They also demonstrated that both abundance and predominant life-cycle observed, changed
when patients were ill either with internal or leukemic diseases. In patients, in contrast to healthy subjects the coliphage numbers were considerably higher and of a substantial fraction of them were virulent phages. In several patients, phage titers were shown to increase with the severity of the clinical symptoms. Recent metagenomic data has also suggested that the majority of all phages present the human gut are temperate, and many are involved in the processes associated with anaerobic gut microbiota.

Impact of animal phages on host bacterial populations. Like the situation described with cyanobacteria, resistance to bacteriophages which infect the chicken gut bacterium Campylobacter jejuni comes at a price. In the chicken gut, phages do seem to exert substantial selective pressure on the population composition of their bacterial hosts, selecting for phage resistance. The cost however is a reduced ability for the bacteria to colonize the gut.

Phages shaping bacterial populations dynamics. Unlike the studies discussed for cyanophages, the impact of naturally occurring phages on bacterial population dynamics has not been quantitatively measured in any animal–associated habitats. However, in some cases there is indirect evidence of the phage pressure. Recently, the intra-species diversity of coliform bacteria in horse gut has been shown to be remarkably high, with over 1000 strains distinguishable by high-resolution PCR fingerprinting, present in a single sample of feces (Fig. 3). A molecular analysis an TEM based analysis of the equine intestinal coliform-coliphage community is consistent with the predictions from of mathematical and experimental modeling of phage-host communities in which the co-evolution of both components is allowed. Field observations indicated that the phages isolated using the indigenous E. coli strains, were able to lyse only 2–8% of the E. coli strains occurring in the same sample (Tarasyan KK, Letarov AV, unpublished data). Again consistent with this is the observation that each indigenous bacterial strain could only be infected by 1, or less frequently 2 bacteriophage genotypes present in the same sample suggesting that there is competition between the viruses for host cells.

There is still much to be learned about phage host dynamics in gut systems, and although there is a high overall density of bacteria for example in the equine intestinal system, most coliphages appear to only infect a small subset of host E. coli strains. The concentration of certain phages ‘types’ has been shown to fall below 10^3 PFU (Plaque forming units) g^-1, and the concentration of their corresponding host cells below 10^6 CFU (colony forming units) g^-1. Under this scenario one would expect that the population of a given phage strain would not be stable and therefore would be eliminated completely, however this is clearly not the case. A further complication is that some bacterial cells may have mechanisms which inhibit bacteriophage intracellular development, causing the extinction of phage lineages. However despite all these theoretical problems posed to bacteriophages, the long term maintenance of some coliphage strains (traced by repeated isolation and genomic DNA RFLP analysis) was observed in the intestine of the horse for more than 2 years (Letarov AV et al., unpublished data).

Possible implications of pseudolysogenic infections. The mechanisms that allow phages to avoid extinction are still not clear; however, a possible explanation may be due to phages from field isolates being able to form pseudolysogenic associations with their hosts. The normally lytic phages may have formed quasi-stable relationships with their hosts. Putative pseudolysogenic infection in E. coli can be observed by growing E. coli cells derived from plaque plaques. When material from plaque plaques is streaked on to fresh Petri-dishes a large proportion of the cells are resistant to the phage whose plaque they were isolated from. This resistance and can be maintained for several passages. In addition to displaying resistance, to the bacteria are able to produce phages active against the parental bacteria strains for between 5–15 passages. These observations lead to the possibility that some phage populations in the horse gut are maintained due to pseudolysogenic micro-colonies or biofilm patches on the surfaces of the food particles and mucosa within the gut environment.

An alternative (or complementary) explanation of the long-time persistence of bacteriophage populations that appear to be severely limited by availability of the hosts was suggested by Kunisaki and Tanji. These authors discovered that some E. coli strains have become phage resistance in anaerobic continuous culture, are resistant because they the bacteriophages used can no longer absorb to them. However approximately 1–2% of the cells grown from these colonies were phenotypic revertants which could be infected by the phage. Interestingly, these cells were not true genetic revertants as authors were unable to isolate phage sensitive derivatives despite screening several hundred sub-clones of the resistant strain. If this observation is confirmed in future experiments, and explanation for it established, it could become a significant milestone in our understanding of bacteriophage ecology in the environments with high density of microbial life. Finally, it is worth mentioning the nascent phage phenomenon whereby phages have broader host ranges immediately after they are released from a bacterial cell than they do after several hours, or longer, have passed.

Summary and future perspectives in phages associated with animals and humans. To summarize our knowledge of phage ecology in the human and animal microflora, the “rules of the game” of the interplay between viruses and the hosts here appear
to be complicated in animal environments because a significant role of the mutual interactions of phage and host within the macroorganism. This game has been going on for hundreds of millions of years and understanding the "rules" is essential if we are to successfully understand the role of bacteriophage in disease, or to exploit them. For example, these natural patterns will inform us as to how we should expect the dynamics of bacteria/phages to stabilize in phage therapy, or in other technologies which use phages to control bacterial populations.

Case Study 3: Archaeal viruses

Are archaeal viruses phages? The final case study discussed in this paper is that of phages in extreme environments, and this necessitates a brief consideration of what we actually mean by a bacteriophage. More than 30 years ago the seminal work of Carl Woese demonstrated that cell based life on this planet was not best described by a dualistic partition into the prokaryotes and eukaryotes. Rather, as now taught by entry level microbiology courses and introductory microbiology texts, living diversity is better described by a division into three domains; the Archaea, Bacteria and Eukarya, each with an associated virus population. There is still a tendency by many to pool viruses associated with the domains Bacteria and Archaea and think of them as bacteriophages. This tendency is deeply and institutionally rooted e.g. by the International Committee on the Taxonomy of Viruses. A visit to the NCBI genomes page www.ncbi.nlm.nih.gov/sites/genome/ provides links to all completely sequenced genomes which can be searched in a variety of ways e.g. within the three domains of cellular life. However, it is not possible to search for viruses infecting cells specifically of the domain Bacteria or specifically of the domain Archaea; these are linked together under 'Phages' and with this in mind they are considered in the final section of this paper.

Archaeal virus discovery and distribution. The domain Archaea was proposed about 35 years ago and the study of viruses associated with it has used classical bacteriophage techniques. Thus far most studies have depended upon lawn/plaque approaches. Since this pioneering work it has become apparent that the Archaea are not just niche players. They may be almost as common as the Bacteria in oceans, soils and subterranean environments and also show enormous diversity. They also occupy and can predominate in so-called extreme environments, such as hot-springs, salt and soda lakes.

Likewise, there is every reason to assume that viruses of the Archaea are as numerous and diverse as those of the bacteria. Reflecting our greater knowledge of the domain Bacteria, almost 6000 bacteriophage have been described, compared with only 50 or so viruses which infect Archaea. In terms of sequenced 'bacteriophages', the NCBI genomes page listed 580 'phages' on June 16, 2010 of which just 32 were viruses of the Archaea, and many of these are sequences of closely related viruses. If we have seen the 'tip of the iceberg' with respect to bacteriophages, then we are just beginning to scratch the 'tip of the iceberg' with viruses of the Archaea. Therefore, our knowledge of their roles in nature is also fragmentary, far more so than for the cyanophages and phages associated with animals. Obviously if we are to begin to understand biogeochemical cycling and environmental ecology then a much more complete genetic census of archaeal viruses in the environment and their effects upon host is essential.

Isolation and characterization of Archaeal viruses. To summarize our current knowledge about these viruses we have to first describe the main divisions within the Archaea. They are divided into two kingdoms, the Crenarchaeota which mainly contains hyperthermophiles, and the Euryarchaeota mainly consists of halophiles and methanogens. Many viruses which infect members of the two kingdoms have been identified. Most viruses have double stranded DNA genomes varying in size from 10 kb to over 100 kb. No viruses of the Archaea with an RNA genome have yet been identified, but it would seem likely that they exist, just as they do in the domains Bacteria and Eukarya.

Around 30 viruses infecting the Crenarchaeota have been described and they are notable not only for generally being isolated from hyperthermophilic hosts growing at temperatures > 80°C but also for an array of unusual morphologies not observed in viruses of the Eukarya or Bacteria. These include spindle shaped virions of the Fuselloviridae e.g. SSV1, infecting members of the genera Sulfolobus and possibly Acidianus, which have small circular genomes from ~15–24 kb. The rod shaped Rudiviridae, SIRV1 and SIRV2 with 35 kb genomes, infect Sulfolobus species. SIFV, an enveloped flexible filamentous virus with a 41 kb genome infecting Sulfolobus is a member of the Lipothrixviridae. These latter two families have recently been grouped into a new order the Ligamenvirales. The droplet shaped virions of the Guttaviridae, Sulfolobus neozealandicus; SNDV, all have circular genomes of around 20 kb. The Ampullaviridae, are exemplified by the Acidianus bottle shaped virion ABV, which has a linear 24 kb genome. The Acidianus two tailed virion of the Bicaudaviridae, Acidianus ATV, has a genome of 62 Kb. This virus is notable for exiting the cell as a lemon shaped fusiform particle which then develops long tails at each pointed end at temperatures above 75°C. This unprecedented extracellular morphological development is entirely independent of the host cell. Spherical viruses ‘the Globuloviridae’ also exist, for example the Pyrobaculum spherical virus PSV. Euryarchaeota predominate in high salt environments, morphological studies again show a diversity of morphotypes with the head and tail variety being in the minority. About 20 viruses have been studied from these environments, infecting members of the genera Halobacteriales, morphologically most resemble head and tail viruses. These are most similar to bacteriophages which infect Bacteria and solely on this morphological basis are classified as Caulovirales including the mycoviruses and siphoviruses. An example of these viruses is the temperate mycovirus Ch1 which has a 58.5 kb linear genome, and infects the haloalkaliphilic host Natrionalba magadii. The lytic viruses HF1 and the closely related HF2, have linear genomes of 75.9 kb and 77.7 kb and infect the haloarchaea Halofaxax lucenstein and Halorubrum corense, respectively. BJ1 with a 43 kb genome infects Halorubrum kocuri. A lytic icosahedral virus SH1 having a linear genome of 31 kb infects Haloarchula hispanica. The Salterprovirus include Hst1 and 2 have linear genomes of...
14.5 and 16 kb respectively and infect Halorubrum hispanicum. The only archaeal virus thus far identified not having a dsDNA genome is the Halorubrum pleomorphic virus 1 which has a ssDNA genome 7048 nucleotides in size. The Euryarchaeotal methanogens also have an identified virus, psi M1 with a linear dsDNA 30 kb genome isolated from Methanothermobacter marburgensis.

Molecular characterization of Archaeal viruses. Genomic analysis of archaeal viruses generally demonstrates very low identity to any other virus sequences. Metagenomic studies over the next few years will rapidly expand our knowledge of these viruses and reveal clues to their biology. Whether the head-tailed viruses of the Archaea currently defined on morphological grounds as Caudovirales are genuinely related to the head-tail viruses of the Bacteria remains to be seen. It has been suggested that the origin of the Caudovirales predates the divergence of the archaeal and bacterial lines. Alternatively, the Caudovirales may have spread from the bacterial to the archaeal domain. Another possibility is that the Caudovirales of the two domains are not evolutionarily related and that any morphological similarities are due to convergent evolution. The study of replication cycles, structure and ecology of the archaeal virus is all still in its infancy, lagging behind the genomic studies and far behind our knowledge of similar aspects with regard to bacteriophages.

Life cycles and population dynamics. Although it has been shown that, archaeal viruses show lytic, temperate and chronic life styles, few studies have looked at the impact that the viruses have on their host diversity and population structure. One fascinating study however has analysed the CRISPRs in 39 strains of the archaeon Sulfolobus islandicus and shown that extensive diversity exists which suggests that multiple strains of archaeal viruses exist within this system. They suggest that the population remaining following phage exposure has multiple resistance mechanisms to the phage they were exposed to. The CRISPRs can "prevent a sweep that that would purge all diversity from the environment." Finally, detailed information about replication and virion structures are only just starting to emerge in archaeal phages and these are all greatly questions for future study. Exciting discoveries of novel biology can be expected.

Conclusions

In all environments phages exist as part of a complex microbial ecosystem which may be either a free living environment such as the ocean, or a microbial environment within a macro-organism.

We have shown how information from isolation, characterization and molecular studies can be combined to build up a picture of phage abundance, diversity and lifestyle. We have also given examples of how this information can show how specific phages influence their host physiology, population dynamics and long term evolution. We also gave examples of how the cost of resistance in either ocean, or gut systems can be significant in terms of growth rates and colonization ability respectively.

The environment in which phages and their hosts inhabit and have evolved in, may have shaped their evolutionary trajectories such as their life cycle and gene content. There is evidence that phages and Bacteria (and Archaea) probably co-existed and evolved together from their onset, while multi-cellular grazers evolved much later in evolutionary time. Under his convincing 'quest for food' argument, Brussow puts forward the hypothesis that this onset of eukaryotes caused bacteria to be attacked from two directions which drove the evolution of lysogeny. It makes sense that where bacteria are associated with eukaryotes, it is advantageous for them to form symbiotic relationships with phages that may boost their ability to survive by encoding toxins and other useful genes.

Certainly phages which don’t form relationships with animals appear to be broadly lytic as discussed here for marine cyanobacteria and those that do often have temperate tendencies such as those found with human coliphages and other gut bacteria. The dynamic between viruses and their hosts does appear to conform to simple predator-prey models in open ocean systems, but not more complex systems. It is clear that both lifestyles of phages are important in moderating different aspects of Bacterial and Archaeal biology. Future work will refine our understanding of the way in which phages control their hosts. The function of the proteins encoded by 'hypothetical' genes in bacteriophage genomes and viromes should also identify novel methods of phage-host interaction. The abundance of particular genes in metagenomic data sets will help direct these studies and determine which genes are the most important to study. Hopefully a fuller understanding of phage dynamics in natural systems will assist in programmes to exploit bacteriophages for example as therapeutic agents with which to control bacterial pathogens.

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