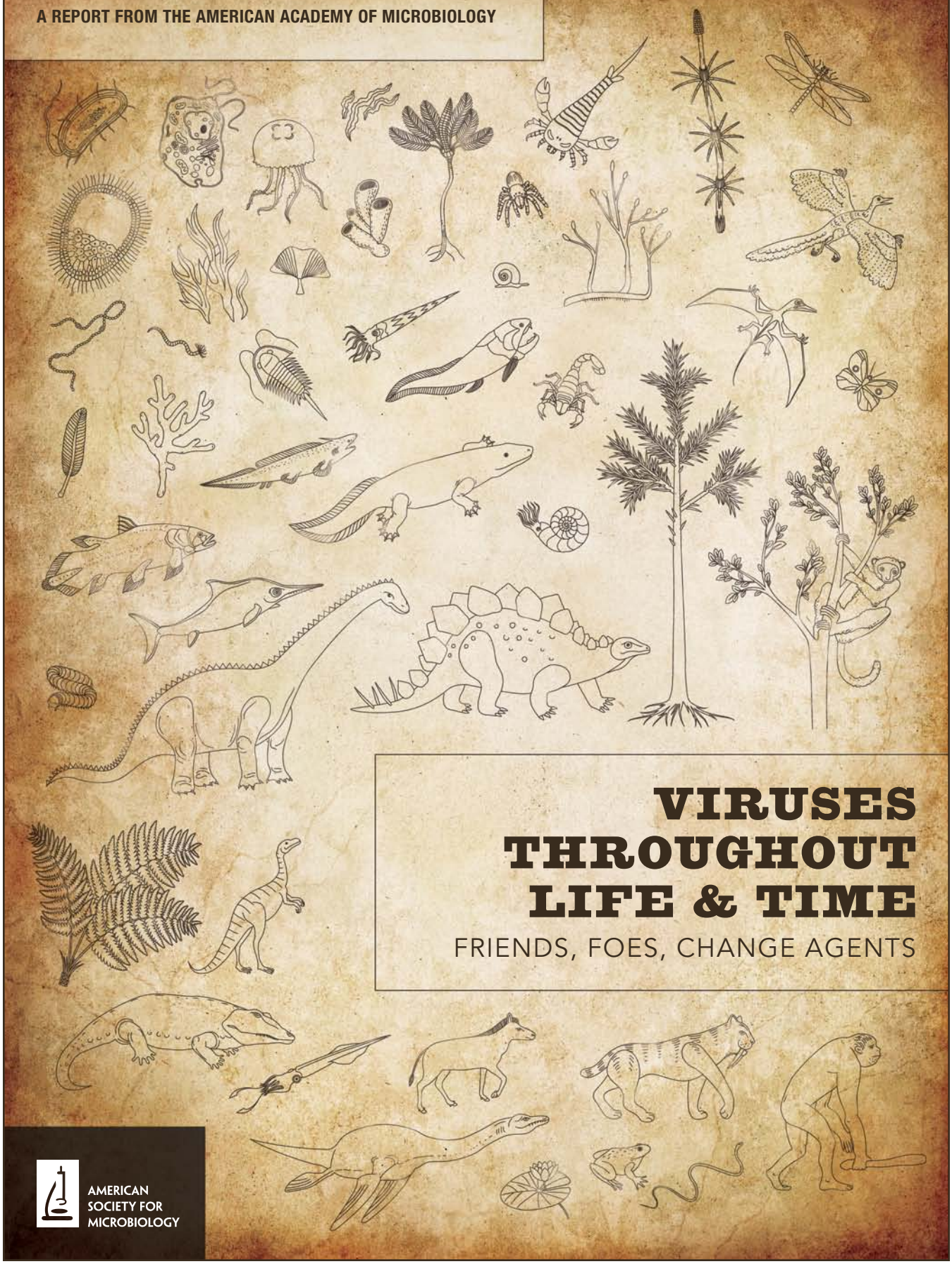
A REPORT FROM THE AMERICAN ACADEMY OF MICROBIOLOGY



VIRUSES THROUGHOUT LIFE & TIME

FRIENDS, FOES, CHANGE AGENTS

AMERICAN SOCIETY FOR MICROBIOLOGY

VIRUSES THROUGHOUT LIFE & TIME: FRIENDS, FOES, CHANGE AGENTS

A Report on an American Academy of Microbiology Colloquium
San Francisco // July 2013

By: Shannon E. Greene and Ann Reid

The American Academy of Microbiology is the honorific branch of the American Society for Microbiology, a non-profit scientific society with almost 40,000 members. Fellows of the AAM have been elected by their peers in recognition of their outstanding contributions to the field of microbiology. Through its colloquium program, the AAM draws on the expertise of these fellows to address critical issues in microbiology.

This report is based on the deliberations of experts who gathered for two days to discuss a series of questions about the roles of viruses in the biosphere throughout the history of life on Earth beyond pathogenesis.

The report has been reviewed by all participants, and every effort has been made to ensure that the information is accurate and complete. The contents reflect the views of the participants and are not intended to reflect official positions of the American Academy of Microbiology or the American Society for Microbiology or the colloquium sponsor.

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Introduction

The microbial world was revolutionized in 1977 when Carl Woese demonstrated that the 16S ribosomal RNA gene could be used to trace the evolutionary relationships among bacteria. Among the revelations of the discovery was that the Archaea, previously thought to be a curious subgroup of bacteria restricted to extreme environments, was in fact an evolutionarily distinct domain of life. Subsequent use of conserved ribosomal gene biomarkers expanded beyond the microbial world to sequence and organize all life on Earth, and revealed that bacteria can be as different from one another as single celled yeast are from humans. Now the relationship of every cellular organism to every other could be pictured in one coherent "tree of life."

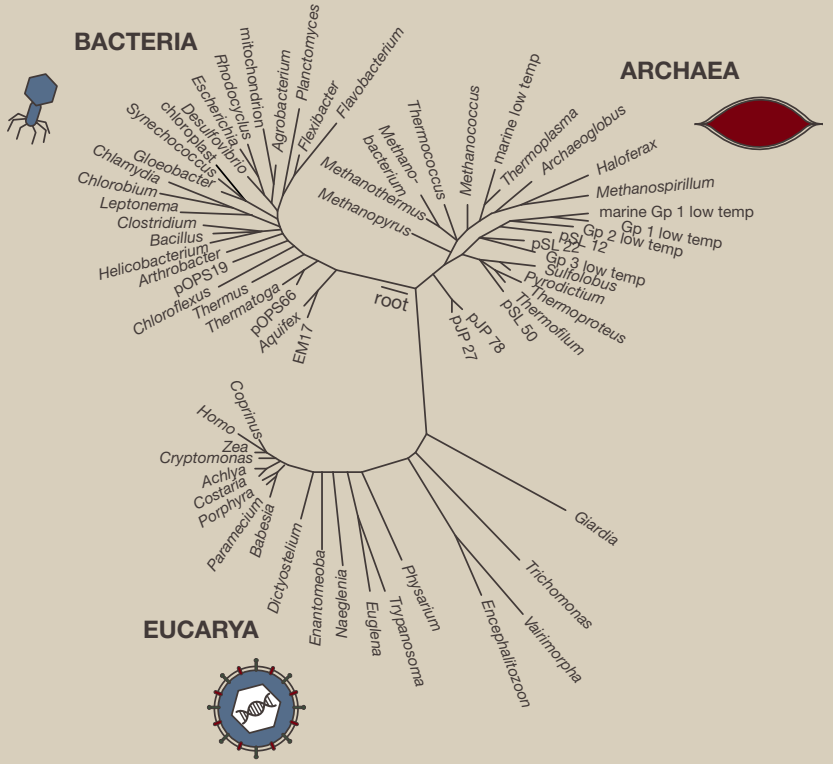
The tree of life, however, excludes one important biological entity, one that is just as varied and extensive as all the other life forms together, if not more. That entity is the virus. We have no "tree" of viruses, but as far as we know, every living organism from bacteria to bonobo, from archaea to algae, can be infected by at least one, and usually many, viruses. As distinct and different as bacteria, archaea, and eukarya are from one another, the three domains of life are linked by their universal ability to be infected by viruses (**Figure 1**). Are they just pests? Parasites? Evolutionary relics? What would happen if all of the viruses on Earth suddenly disappeared?

We have no "tree" of viruses, but as far as we know, every living organism from bacteria to bonobo, from archaea to algae, can be infected by at least one, and usually many, viruses.

Without viruses, life on Earth would have been very different, or perhaps there would be no life at all.

FIGURE 1:

Use of ribosomal RNA genes and other highly conserved markers enable construction of phylogenetic trees illuminating the three domains of the tree of life: bacteria, archaea, and eukarya. Although each domain contains widely diverse members, viruses can infect all domains. Representative virus forms are depicted, although viruses infecting each domain can exhibit diverse morphologies.



Adapted from FAQ: Human Microbiome (2013)

Most people would likely welcome such a scenario, as viruses are known primarily as pathogens, and the public sentiment is generally one of fear and loathing. Many might assume that if viruses were absent, the impact on life on Earth would be minimal or perhaps even positive. But in fact, viruses play numerous crucial biological roles at multiple scales, from individual cells to entire ecosystems. Without viruses, life on Earth would be very different, or perhaps there would be no life at all.

In July 2013, the American Academy of Microbiology convened 25 virology experts to discuss the myriad roles of viruses in the natural world which advances in technology are rendering increasingly amenable to detailed study. Colloquium participants included experts studying topics straddling the field of virology and other disciplines such as evolution, ecology, and climatology that are affected by the viral world. The colloquium focused not on viruses as simple disease agents, but rather on the complex interactions between viruses and their hosts, and how these interactions influence the world around us and all life on it. The Academy would like to thank the Gordon and Betty Moore Foundation for supporting this colloquium.

The essential role of viruses in biological systems extends back through time to the earliest years of life on Earth.

For the first two billion years of life, Earth was a microbial planet. It is commonly accepted that viruses and microbes dominated the biological landscape. But where did viruses come from? Which came first, the virus or the cell? The question of the origin of viruses has profound implications for how we understand biology today, and any answers suggest mechanisms for the origin of life on Earth.

The current scientific consensus is that life originated in an RNA world. RNA molecules would have encoded the earliest enzymatic processes and genetic information. While improvements on those activities could have been achieved through a high incidence of mutation, those improvements would have also been difficult to maintain because of those same high mutation rates. Thus, it has been proposed that a transition to a DNA-based method of storing information would have helped to keep records of the original genetic code in a more stable form.

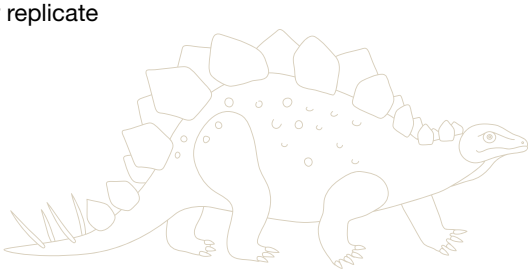
So then, did cellular life arise from the primordial soup of nucleic acids, lipids, and proteins and then degenerate into viruses? Or did viruses give rise to cellular life forms?

IF THE CELL CAME FIRST...

By definition, a virus is an entity that replicates within cells, thus implying that the cell had to come first. To date, no self-replicating or completely free-living virus has been identified. The nearest example to a free-living virus is a recently discovered archaeal virus that grows two tails at each of its ends following its release from the host (Haring *et al*. 2005). However, such examples are extremely rare and most viruses are highly dependent on their hosts for replication. Very large viruses such as the Mimiviruses, encode tRNA genes, a feature unusual for viruses and potentially indicative of reductionist evolution from a self-sufficient cell.

IF THE VIRUS CAME FIRST...

Viruses could represent a stepping-stone from non-life to life. The original viruses could have been predatory entities that subsequently lost the ability to self-replicate, similar to endosymbiotic bacteria, which no longer replicate outside of their hosts but are derived from free-living ancestors.



HOW WERE VIRUSES DISCOVERED?

The word “virus” itself comes from the Latin meaning “poison.” Historically, viruses were identified for their role as pathogens. Tobacco mosaic disease (TMV) was shown in 1866 to be transmitted between plants in much the same way as bacterial infections. However, infected sap still proved infectious even when filtered through pores smaller than bacteria. In 1898, Martinus Beijerink showed that the infectious agent was not a poison or toxin, but rather a non-bacterial agent capable of reproducing and multiplying within tobacco cells. Beijerink’s “virus,” TMV, was purified and crystallized by Wendell Stanley in 1935 and finally visualized by electron microscopy in 1939.

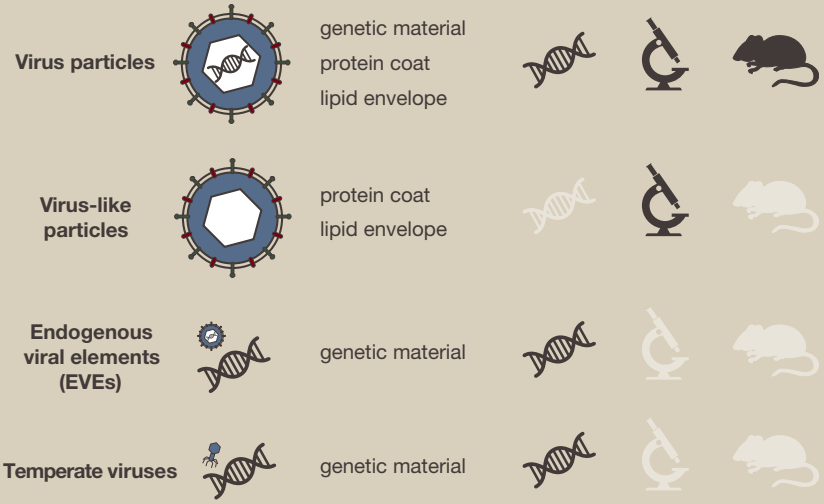
WHAT IS A VIRUS?

Viruses are microscopic entities that have the same genetic material as all other life forms — DNA and RNA — but depend upon the machinery of host cells to produce more viruses. Beyond this unifying feature of dependent reproduction, viruses are incredibly diverse. Not all viruses have the same genetic material, and not all viruses exist as particles seen with microscopy. The viral world can be largely grouped into four categories (**Figure 2**):

1. **Virus particles** or **virions** are comprised of (i) genetic material, either DNA or RNA; (ii) a protein coat to protect the genetic material; and, in some cases, (iii) a lipid envelope to surround the protein coat. Virions can be quantified visually by electron and epifluorescence microscopy.
2. **Virus-like particles** (VLPs) look like viruses, but do not contain any genetic material. Such particles have been observed and isolated from Hepatitis B patients for over 40 years (Bayer *et al*. 1968). VLPs are problematic when assessing viral numbers, as they look like ordinary viruses under the microscope and yet are noninfectious.
3. **Endogenous viral elements** (EVEs) consist of DNA sequences in host genomes that are derived from previous viral infections of germline cells (Katzourakis and Gifford 2010). EVEs are typically the result of retroviral infections, as integration in host genomes is a required step in the retroviral life cycle. EVEs can also derive from parvoviruses, filoviruses, bornaviruses, and circoviruses among others.
    a. In some cases, particularly among retroviruses, the entire viral genome is inserted in the host genome, resulting in a **provirus** that can still be capable of producing infectious particles or further proliferations of viral sequences in the germline. Most mammalian species harbor hundreds of thousands of endogenous retroviruses in their genomes.
    b. More frequently, only fragments of the viral genome remain after infection. This is especially true for viruses for which integration is not a normal part of the replication cycle.
    c. EVEs can only be detected through nucleic acid sequencing, although it can be difficult to distinguish between endogenized viruses and non-viral selfish genetic elements such as LTR retrotransposons in invertebrate animals (Malik *et al*. 2000).
4. **Temperate viruses** are to bacteria and archaea what proviruses are to eukaryotes. A temperate bacteriophage either integrates its nucleic acid in the bacterial genome or maintains itself as an extrachromosomal plasmid in the cytoplasm. The **prophage** is then transferred to daughter cell progeny upon replication of the bacterium. Temperate viruses, like EVEs, can only be detected through nucleic acid sequencing.

FIGURE 2:

Viruses can be broadly grouped into four classes: virus particles, or virions; virus-like particles; endogenous viral elements, or EVEs; and temperate viruses. Based on the physical characteristics of these groups, virologists can detect the presence of viruses in different ways. Virions, EVEs, and temperate viruses can all be detected through nucleic acid sequencing; however, EVEs and temperate viruses only exist as genomes or remnants of genomes and thus cannot be seen through microscopy. Virions and virus-like particles can both be seen through microscopy, and only virions can be detected in an infection setting.



Scientists contemplating a viral origin of life on Earth base their hypothesis on a few key observations:

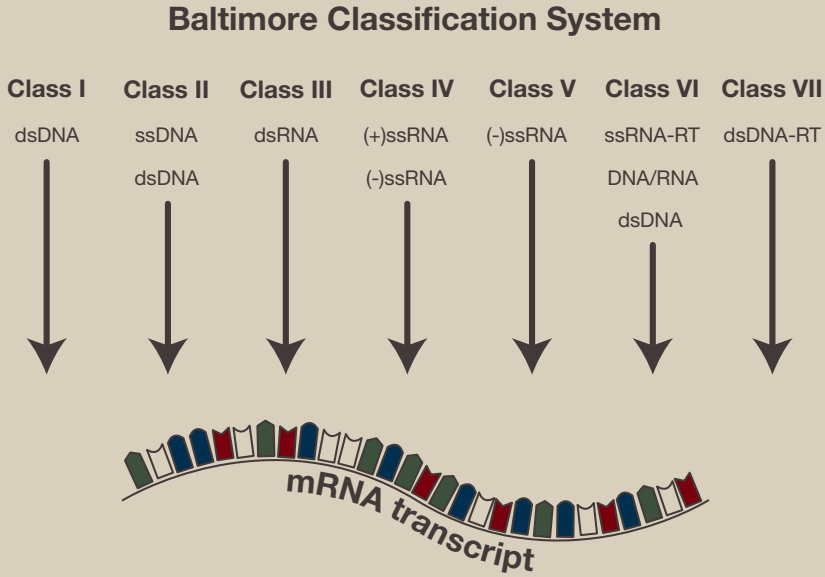
1. First, there is no evidence that all modern viruses share a common ancestor — viruses have many different mechanisms of replication, suggesting multiple origin events (**Figure 3**).

2. By contrast, all cellular life forms including bacteria, archaea, and eukarya, are thought to share a universal common ancestor. These organisms also all use a single DNA-based mechanism of replication.

3. Finally, cellular and viral proteins show very little similarity, suggesting that viral genetic novelty is either extremely ancient or arose in cells that no longer exist. Viral genetic material is not likely to have simply escaped from ancestors of currently known cells and been encapsidated by structural proteins.

FIGURE 3:

The Baltimore classification scheme groups viruses based on genome type and replication strategy. For a virus to replicate, it must generate mRNAs from its genome and there are seven ways of doing so, or seven classes of viruses. Class I viruses can transcribe mRNA directly from their double-stranded (ds) DNA genomes, while Class V viruses must use an RNA-dependent RNA polymerase to transcribe their negative sense single-stranded (ss) RNA genomes. Class VI and Class VII viruses both make use of reverse transcriptase (RT) enzymes in the replication cycles of their ssRNA and dsDNA genomes, respectively.



AN ALTERNATIVE SCENARIO...

An alternative scenario involves the concurrent evolution of both viruses and cells; a single lineage could have branched into two, or perhaps the two forms experienced independent but concurrent emergence events. A variety of self-replicating entities could have diverged, giving rise to one cell-like and several virus-like lineages that subsequently coevolved.

It is probable that a combination of these scenarios happened. Some viruses and their genes could have come from cells, and other viruses could have given rise to cells. What is clear is that viruses are, and probably always have been, an integral and ubiquitous part of all biological processes.

What were the ancient viruses and cells doing?

Colloquium participants emphatically agreed that any understanding of early Earth ecology must include consideration of viruses.

To reconstruct early Earth biology, scientists turn to phylogenetic reconstructions of highly conserved protein families, presumed to have been among the earliest to evolve. Many of these proteins turn out to have thermophilic catalytic functions; they are simpler, slower, and have fewer domains than more recently evolved varieties (Ingles-Prieto *et al*. 2013). Early Earth proteins may well have acted like simple catalysts. Any surviving evolvable proteins likely contained stable backbones or active sites with flexible or floppy regions that were amenable to tinkering, enabling the early proteins to interact with each other and other molecules.

With high mutation rates and extensive replication capabilities (viral polymerases can exhibit mutations rates up to 1 in 10,000 base pairs and some viruses can produce over 1,000 progeny per cell they infect), viruses could serve as a ready reservoir of genetic material to increase variety in these early proteins. The transition from a more evolutionarily dynamic RNA-based world to a more stable DNA-based information storage mechanism would have slowed down evolution rates. However, retaining RNA as the messenger molecule between the genetic library of DNA and functional proteins enabled early cells, and all cells thereafter, to interact genetically with both DNA and RNA viruses. The flexibility offered by viruses as external genetic elements would have helped early cells to supplement their existing capabilities and explore the available genetic sequence space in new ways.

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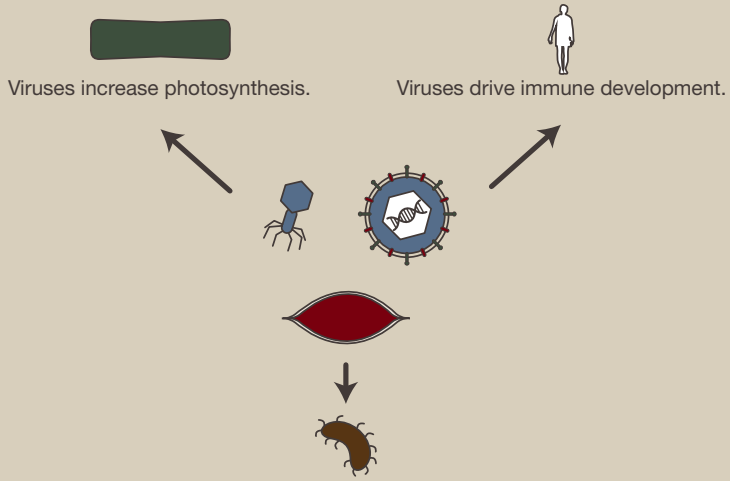
As movers of genetic material and epigenetic elements, viruses would have had the potential to rapidly expand and influence the genomes of their hosts. Indeed, viruses can and do act as epigenetic elements for their hosts today. Plants carry many cytoplasmic double-stranded RNA viruses that have been passed down vertically for thousands of years and are evolutionarily very difficult to eliminate. These viruses may be influencing gene expression in their hosts, or may themselves express proteins important for the host (Roossinck 2012).

Perhaps one of the most critical evolutionary roles played by viruses is the result of continual skirmishes between viruses and the organisms they infect. The evolutionary arms race between viruses and their hosts has led to ever more sophisticated immune systems among eukaryotes including both innate and adaptive immune systems. The realization in the last 20 years that all multicellular organisms have long-term, beneficial relationships with communities of bacteria (known as an organism's microbiome) has led to an appreciation of the immune system as not purely defensive, but also important for communication with the beneficial microbes. It seems likely that there are both positive and negative interactions between viruses and the host immune system. Either way, it is clear that cross talk between viruses and the immune system has been an important evolutionary driver.

FIGURE 4:

All the world's a stage and viruses are major players. Viruses participate in essential Earth processes and influence all life forms on the planet, from contributing to biogeochemical cycles, shaping the atmospheric composition, and driving major evolutionary developments.



Early Earth events are very remote and are primarily accessible through examining the fossil record for geochemical clues for biological activity. These events have preserved morphological signatures and have reconstructed evolutionary history through the phylogenetic analysis of existing organisms. Viruses are virtually invisible by either of these methods and so their role in evolutionary biology has been largely ignored. While viruses leave little trace of their activity in the fossil record, they did leave their mark and most likely participated in key events in early Earth history (**Figure 4**).

1. VIRUSES COULD HAVE ACCELERATED EARLY CARBON CYCLES.

Early life forms were most likely *chemolithoautotrophs*, deriving their chemical energy from the rocks and oceans around them and building cellular components from carbon dioxide fixed directly from the environment. Anoxygenic phototrophs likely abounded on the early Earth, making a life for themselves with sunlight and more carbon dioxide. Bacteria today engage in an enormous variety of additional metabolic activities, and many of the species familiar to humans, like those that cause human disease (*Staphylococcus* and *Salmonella*) or contribute to the foods and beverages we enjoy (cheese and wine), are *heterotrophs*. That is, these species use complex carbohydrates or other organic molecules for both cellular energy and building cellular components, independent of rocks or sunlight as energy sources. Where did the earliest heterotrophs find this wealth of organic carbon to exploit? The bacterial and archaeal cell deaths resulting from viral infections would have released massive amounts of organic carbon into the oceans, generating a specific niche for heterotrophs to exploit while adding new steps to the carbon cycle.

2. VIRUSES INCREASE PHOTOSYNTHESIS RATES AND COULD HAVE ENHANCED THE OXYGENATION OF THE ATMOSPHERE.

Geochemical evidence points to an atmosphere devoid of molecular oxygen until about two billion years ago, when oxygenic photosynthesis rose to prominence. The step-wise increase from trace levels of free oxygen to today's 21% is called the Great Oxidation Event (Lyons *et al.* 2014). This early example of biologically driven atmospheric change was accomplished largely by cyanobacteria, which are still some of the most common organisms on Earth. Today, photosynthesis in the world's oceans is largely driven by the two most common cyanobacteria, *Prochlorococcus* and *Synechococcus*, which together account for 25% of carbon fixation on Earth (Flornbaum *et al*. 2013). Part of their photosynthetic activity, however, may be attributed to viral partners — cyanophages. Many cyanophages — viruses that infect cyanobacteria — encode photosystem II reaction core proteins. The psbA gene encodes one of these proteins and is the most rapidly turned over photosynthesis core protein in all oxygen-yielding photosynthetic organisms. Maximal viral production is dependent upon photosynthesis rates, so expression of these photosynthesis genes during infection enhances not only photosynthesis in the host, but also cyanophage replication (Sullivan *et al*. 2006).

Because *psbA* is so abundant among marine cyanophage — it has been detected in 88% of cyanophage genomes sequenced (Sullivan *et al*. 2006) — the evolutionary relationship between cyanophage and photosynthetic capacity of their hosts could have been an ancient driver of oxygenic photosynthesis and the global rise of molecular oxygen in the atmosphere.

3. VIRUSES COULD HAVE DRIVEN THE ORIGIN OF THE EUKARYOTIC CELL.

The origin of the eukaryotic cell is shrouded in mystery with several proposed theories. Eukaryotes distinguish themselves from bacteria and archaea primarily in their structural elements — namely, their membrane-bound intracellular organelles that take on specific tasks within the cell. Although examples of membrane-bound organelles are coming to light in some bacteria, possession of a true nucleus and mitochondria are unique hallmarks of a eukaryotic cell. Eukaryotes share many similarities with archaea including membrane composition, and their mitochondria are now commonly accepted to have derived from an endosymbiotic alpha-proteobacterium. But what was the identity of the proto-eukaryote that engulfed the bacterium? And where did the nucleus come from? The hypothesis of viral eukaryogenesis includes a role for all three early life forms: a bacterium, an archaeaon, and a virus. This hypothesis proposes that the cell nucleus of the primitive eukaryotic cell derived from a large DNA virus that became an endosymbiotic partner with a methanogenic archaeon, with the new cell being powered by bacteria-turned-mitochondria (Bell 2009).

In an alternative scenario, the original eukaryotic lineage might have provided a bacterium some relief from viral predation. In this scenario, replacement of the cell wall with a multi-functional membrane might have enabled the newly flexible cell to phagocytose viruses as both infection prevention and potentially as a food source.

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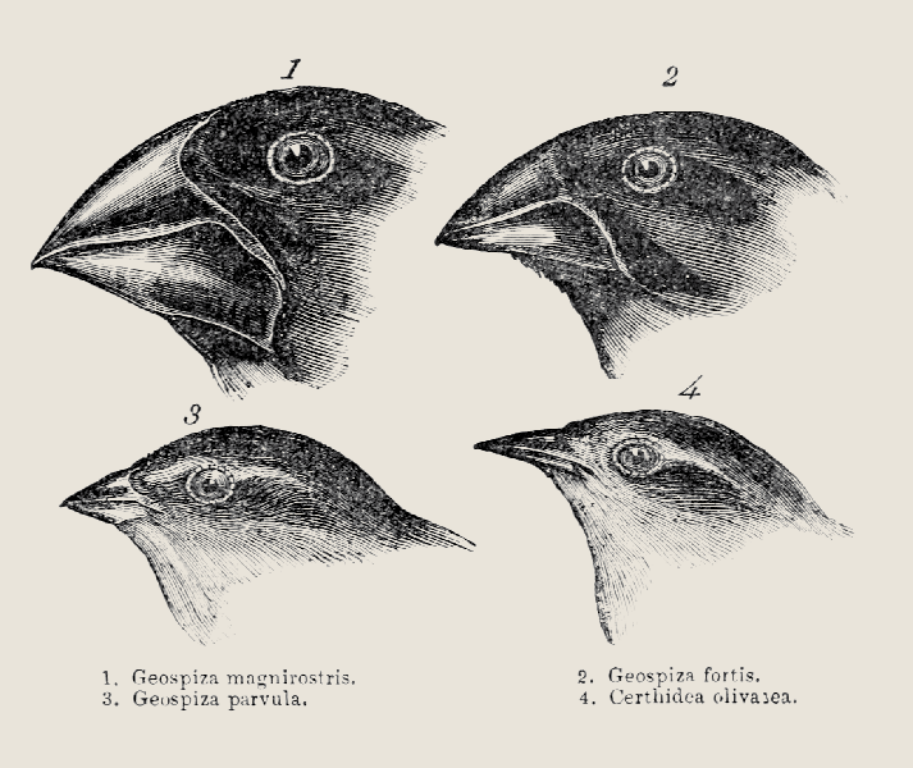
Viruses have had immense potential to act as drivers of major speciation events throughout Earth's history, both expanding and contracting evolutionary space.



4. MULTICELLULAR ORGANISMS COULD HAVE ARISEN AS A RESPONSE TO VIRAL PREDATION.

Like the eukaryotic cell, multicellularity also might have been fueled by viral predation. While in lean times, cells might favor motility and a solo lifestyle to enable their search for food, in times of nutrient richness, group living might have provided a respite from infection (Ruardij *et al*. 2005).

The take home message is that viruses have been and are major drivers of evolution. There may be no selection pressure on Earth greater than viruses. Clearly, viruses have had immense potential to act as drivers of major speciation events throughout Earth's history, both expanding and contracting evolutionary space. While viruses drive genetic innovation by shuffling existing genes and providing rapidly evolving new ones, infecting viruses might also place constraints on what an organism can do if it is to defend itself against its viral foes.

Viruses have been and continue to be drivers of evolution.

Viruses exert selective pressure on all life forms — no species is immune. Through their multitude of activities, viruses shape the evolution of other organisms through three major routes, two of which act directly on their host, and one of which uses external pressures, to be discussed in a later section.

The direct mechanisms by which viruses exert selective pressure on their hosts are pathogenicity and introducing new traits epigenetically or through lateral gene transfer.

PATHOGENICITY AS A DRIVER OF IMMUNE EVOLUTION

Infection by viruses is a powerful force shaping hosts defense mechanisms, which in turn forces viruses to counter with immunity-evading tricks. The struggle between pathogen and host results in antagonistic coevolution. Even organisms as seemingly simple as bacteria have evolved mechanisms of evading viral predation; indeed, microbes have complex immune systems. Some of these mechanisms are innate, such as cell surface modifications to prevent phage entry or restriction-modification systems to digest phage nucleic acids, while others are adaptive such as the CRISPR-Cas system of bacteria and archaea. Indeed, 40% of bacteria and 90% of archaea encode Clustered Regularly Interspaced Short Palindromic Repeat sequences (CRISPRs) that provide resistance to exogenous genetic elements. The repetitive sequences, for which CRISPR immunity is named, are interspersed with short sequences called spacers that are identical to sequences from plasmids and phage. RNA transcribed from the spacer elements can form a complex with Cas proteins to recognize and cleave complementary target RNAs encoded by invading viruses, thus halting viral replication. The CRISPR-Cas system “learns” and adapts to new phage infections by incorporating new phage sequences into the repetitive CRISPR genomic loci (Sorek *et al*. 2013), and bears similarities to the plant adaptive immune system of RNA interference.

Phage have evolved mechanisms to counteract even the sophisticated immunity of their bacterial targets. Anti-CRISPR genes were recently detected in several phages infecting *Pseudomonas aeruginosa* (Bondy-Denomy *et al*. 2013) and a phage infecting *Vibrio cholerae* actually encodes its own CRISPR-Cas system that targets a putative phage-inducible chromosomal island (PICI) in the *V. cholerae* genome (Seed *et al*. 2013). Phage infection triggers the PICI to inhibit phage activity through an unknown mechanism; the phage, in turn, use their own CRISPR-Cas systems to counteract the *V. cholerae* defense.

Upon infection of a new host, bacteriophage can transfect the hitchhiker DNA into that cell.

Use of RNA as a tool for defense is not limited to bacteria and archaea. RNA interference, or RNAi, is used by plants and many other eukaryotes including animals. The enzyme Dicer cleaves cell-transcribed double-stranded RNAs into smaller fragments of about 20 nucleotides. These small RNAs bind to complementary sequences from infecting viruses, and the associated RNA-induced silencing complex cleaves the target. Some plants even have multiple RNAi systems, each responding specifically to different virus exposures (Blevins *et al*. 2006). However, like phage, plant viruses are also adept at evading or suppressing the RNAi pathway in their hosts. *Cucumber mosaic virus*, for example, evades RNAi via a virulence factor that localizes to the nucleus and binds directly to host double-stranded and single stranded RNAs (Lucy *et al*. 2000; Goto *et al*. 2007).

Apoptosis, or regulated cell death, may have evolved as a primordial reactionary mechanism to shut down viral replication in an infected host. The apoptotic response is common to all metazoan phyla and can also trigger a subsequent immune response to alert the host to the invasive threat. Some viruses co-opt apoptosis signaling cascades for their own replication or immune suppression purposes. For example, apoptotic killing of HIV-infected macrophages is prevented by the viral envelope glycoprotein, which induces a pro-survival cytokine (Swingler *et al*. 2007).

HORIZONTAL GENE TRANSFER INTRODUCES NEW TRAITS — BOTH PATHOGENIC AND BENEFICIAL

Bacteriophage are especially adept at facilitating movement of genomic material among bacteria. During a lytic life cycle, a bacteriophage may package pieces of the bacterial chromosome or plasmid DNA in addition to or instead of its own genetic material. Upon infection of a new host, bacteriophage can transfect the hitchhiker DNA into that cell. Some of the DNA will be degraded and used for cellular metabolism, while plasmids can recircularize, and other pieces of DNA, if they share similarity with sequences in the new host chromosome, may recombine and become incorporated into the genome. Even phage-like elements, such as Gene Transfer Agents (GTAs), are capable of transferring random pieces of host bacterial genome from one cell to another; however, the DNA carried is not sufficient to encode the GTA structural proteins themselves and thus GTAs cannot replicate like true phage (Lang *et al*. 2012).

Antibiotic resistance genes can be transferred between bacteria via bacteriophage, as can virulence genes. Indeed, bacterial endotoxins are almost exclusively encoded by bacteriophage. Bacteria can become addicted to these mobile genetic elements when they encode both the toxin and the antitoxin. For example, the causative agent of cholera, *Vibrio cholerae*, owes its virulence to its eponymous cholera toxin, introduced by horizontal gene transfer. Virulent strains of *V. cholerae* carry a variant of temperate, or lysogenic, bacteriophage called CTX. As a temperate phage, CTXϕ integrates into the *V. cholerae* genome and brings with it the genes *ctxA* and *ctxB* encoding the cholera toxin. During human infection, phage particles are secreted from host bacteria without killing the cells, thus enabling their spread to new *V. cholerae* hosts (Faruque and Mekelanos 2012).

Similarly, *Salmonella enterica* serovar Typhimurium produces two superoxide dismutases that protect the bacteria from oxidative stress inside macrophages. One of these superoxide dismutases, SodCI, is regulated by a virulence signal

transduction system and was horizontally transferred to *Salmonella* via the Gifsy-2 phage (Golubeva and Slauch 2006). The phage gene *sodCI* has become part of the PhoPQ virulence signal transduction system. *sodCI* and other genes in the PhoPQ regulon are up-regulated in response to the low pH and antimicrobial peptides encountered when *Salmonella* lives inside macrophage vacuoles. Infected macrophages release an oxidative burst to try to kill the bacteria, but the phage protein product counteracts the superoxide produced.

However, not all of the traits conferred by viruses increase the pathogenicity of their hosts. In many instances, the evolutionary history of virus and host is so intertwined that the viral genetic code becomes incorporated into its host's genome, conferring novel traits. Endogenous retroviruses (ERVs) are genetic elements derived from free-living (exogenous) retroviruses. When retroviruses infect germline cells, their genetic sequences might be passed on to host offspring as a novel allele. Vertebrate genomes are particularly rich in endogenous retroviral sequences, and in fact, ERVs make up 8% of the human genome. Because ERV elements integrate into host genomes with their own promoter elements, they can influence the expression of nearby genes and also modify the methylation state or histone architecture of the genetic region (Sharif *et al*. 2012). The differential gene regulation can be sensitive to new environmental signals, generating phenotypic plasticity in the host animal.

Endogenous retroviruses can also protect their hosts from environmental pressures — predation, extreme temperatures, or infection by other viruses. For example, while active Jaagsiekte sheep retrovirus (JSRV) is the causative agent of a contagious lung cancer in sheep, its endogenous form plays several key roles in domesticated sheep, including conferring protection against active JSRV infections and playing a role in placental morphogenesis (Arnaud *et al*. 2008). Now, numerous non-retrovirus elements have been found in genomes too, even from viruses that have only a cytoplasmic RNA lifestyle (Horie *et al*. 2010).

VIRUSES AND THE MAMMALIAN PLACENTA

The evolution of the placenta in mammals seems to have been driven by viruses not just once, but six or more times. The mammalian placenta is a multifunctional exchange organ that enables nutrient delivery, waste elimination, and gas exchange between the mother and developing fetus. Cells at the placental/uterine interface fuse into a single layer called the syncytiotrophoblast, essential in allowing the fetus to obtain nutrients from its mother. A protein critical for the fusion process, syncytin, is actually derived from an endogenous retroviral locus that became "domesticated" (Mi *et al*. 2000).

Syncytin analogs are found throughout mammalian genomes, but sequence evidence suggests that this important gene was acquired by diverse mammalian lineages on at least six independent occasions — including the primates, muroids, leporids, carnivores, caviids, and ovis (Dupressoir *et al*. 2012). ERVs not only contributed syncytins to the mammalian placenta, but also brought enhancer elements to drive gene expression and have led to diversification of placental development (Chuong *et al*. 2013).

The idea of viruses as commensals is so new that there are as yet few well-characterized examples.

Viruses as symbionts

Through diverse symbiotic relationships across multiple trophic levels, viruses continue to affect the fitness and survival of organisms across the tree of life.

Beyond their roles as pathogens, viruses also participate in many commensal partnerships with other organisms, often conferring ecological adaptations or immunity to various threats. Some of the interactions are transient, others evolutionarily persistent. The idea of viruses as commensals is so new that there are as yet few well-characterized examples. Now that it is clear that such commensal relationships exist, no doubt more will be discovered.

1. VIRUSES INCREASE THEIR OWN REPRODUCTIVE FITNESS BY BOOSTING HOST FITNESS.

Viruses frequently amend their hosts' traits or behavior to further their own replication and spread. The virus known as "*tulip breaking virus*" benefited from a very human-centric replication strategy, as the infection phenotype is one of dramatic streaks and flame-like markings on the tulip petals. Such streaks were deemed so attractive that 17th century breeders in Holland grafted infected bulbs onto plain tulip plants, furthering the spread of this virus. In the wild, aphids transmit tulip-breaking virus from plant to plant as they feed. Whether the dramatic changes in petal coloring are merely a byproduct of infection or a means of making the host plants more attractive to feeding aphids is still unknown (Lesnaw and Ghabrial 2000).

Not all viruses rely on human intervention for their survival and spread; others improve the competitive fitness of their hosts against close relatives. Yeast strains compete against one another in the wild, and some use viral toxins to do so. The killer yeast phenotype requires two viruses — a helper virus and the toxin-coding killer virus, which depends upon its helper for stable maintenance and replication (Schmitt and Breinig 2006). Thus, a team of viruses allows their particular yeast strain to increase its fitness over related strains, simultaneously producing more viral progeny.

2. VIRUSES INFLUENCE HOST BEHAVIOR TO SPREAD.

Viral modification of the host is not always mutually beneficial. In one of the more sinister symbioses, a species of baculovirus manipulates the behavior of its gypsy moth host. Expression of a single viral gene *egt* forces the infected gypsy moth caterpillar to climb to treetops in daylight, making them particularly vulnerable to predators. Behavior modification is driven by expression of a specific viral protein that inactivates the molting hormone 20-hydroxyecdysone (Hoover *et al*. 2011). The virus additionally produces cathepsin-like protease and chitinase enzymes that digest the moth body into a virus-filled liquid, raining as many as 10 million viral occlusion bodies per milligram of larval tissue down upon uninfected caterpillars below (Clem and Passarelli 2013).

Rabies viruses are notorious for their host behavior modification. Encephalitic rabies causes neurological symptoms such as hydrophobia, delirium, aggression, disorientation, and seizure activity among others. Along with high viral titers in the salivary glands, such behavioral traits magnify the risk of viral spread by increasing violent confrontations between infected and uninfected animals.

3. VIRUSES PARTICIPATE IN MULTITROPHIC SYMBIOSES.

A persistent and ancient example of viral-host association is that of viral and fungal mutualistic symbionts conferring heat tolerance to the panic grass that grows in the hot soils of Yellowstone National Park. Indeed, a panic grass *Dichanthelium lanuginosum* can only survive in soils greater than 50°C if it carries the endophytic fungus *Curvularia protuberate* that is itself infected by a viral partner (Márquez *et al*. 2007).

Everywhere virologists look, viral persistence and mutualistic symbioses of many types can be found (Roossinck 2011):

i. **Polyadnaviruses permit parasitic wasp offspring survival.** These viruses replicate in the reproductive tissues of female parasitic wasps and are injected along with the wasp egg into the body of a host caterpillar. While the caterpillar's immune system would normally recognize the deposited egg as a foreign body and prevent its development, the virus instead modulates the immune response so that the wasp egg can develop unmolested (Edson *et al*. 1981).

Unfortunately for vertebrates, mosquitos are more adept at finding blood vessels in hosts infected with parasites such as malaria or Rift Valley fever virus, thus enabling more efficient transmission of the pathogens.

ii. **Bacteriophage provide protection from parasitism.** By contrast, the bacteriophage APSE of the bacterium *Hamiltonella defensa*, itself an endosymbiont of aphids, actually protects the aphid host from parasitic wasps (Oliver *et al*. 2009). Aphids infected only with *H. defensa* are significantly more vulnerable to wasp parasitism than aphids infected with the *H. defensa*-APSE duo.

iii. **Plant viruses increase the fecundity of their insect carriers.** Tobacco curly shoot virus (TbCSV) is transmitted by whiteflies, which in China exist in both native and invasive B subtypes. Unfortunately for farmers, the fecundity and longevity of the invasive B whiteflies increase 12 fold and 6 fold, respectively, when feeding on infected tobacco plants (Jiu *et al*. 2007). The mutual benefits between invasive B whiteflies and TbCSV may eventually lead to displacement of indigenous insects and outbreaks of plant disease.

iv. **Bacteriophage enhance virulence, protect secondary hosts, and eliminate third party species in a tangled web of interactions.** A bacterial pathogen of rye grass, *Rathayibacter toxicus*, produces bacteriophage-encoded corynetoxins that are among the most lethal natural toxins and present grave danger to livestock grazing upon rye grasslands. *R. toxicus* moves between host plants using nematode vectors which are also toxin sensitive. Regulation of toxin production is controlled by the bacteriophage to permit transmission of the bacteria (and phage) from host to host (Ophel *et al*. 1993). Thus, the bacteriophage are mutualistic to *R. toxicus*, parasitic to nematodes and grazing animals, and mutualistic to rye grass.

v. **Pathogens enhance their own transmission by increasing attractiveness of their hosts to insect vectors.** Unfortunately for vertebrates, mosquitos are more adept at finding blood vessels in hosts infected with parasites such as malaria (Lacroix *et al*. 2005) or Rift Valley fever virus (Turell *et al*. 1984), thus enabling more efficient transmission of the pathogens. Enhanced mosquito feeding has been linked to increased body temperatures or carbon dioxide emissions from febrile hosts (Kuno and Chang 2005.) Viruses may also affect the behavior of their vectors; arbovirus-infected mosquitos feed longer and more often (Lefèvre and Thomas 2008).

   A similar story exists for plant viruses, where infected plants attract more aphid vectors (Ingwell *et al*. 2012, Moreno-Delaguete *et al*. 2013).

vi. **Bacteriophage protect hosts by modulating gut microbiota.** In mice, bacteriophage in mucus lining the gut bind and kill bacteria; ultimately the metazoan is the beneficiary while the bacteriophage gain microbial hosts in which to replicate. Because phage target their bacterial prey in a species-specific manner, they can shape gut community structure (Ventura *et al*. 2011). Phage can also participate in dissemination of antibiotic resistance genes and increase the adaptive capacity of intestinal pathogens (Sun and Relman 2013), and thus their presence is not always mutualistic with the host.

Advances in technology allow viruses to be detected in all environments.

Biologists are now beginning to appreciate the role of microbes in complex communities, and can no longer consider organisms in isolation. Rather, life should be viewed as a series of nested ecosystems in which the organism represents the first layer — an ecosystem in and of itself made up of a host and its microbial symbionts, together known as the holobiont.

This ecosystem comprises the sum of the genetic information of both the host and its symbiotic microorganisms. While much of the research investigating the microbial partners of humans, plants, corals, and other organisms to date has focused on bacteria, archaea, and microscopic eukaryotes, the viral component should not be ignored. The role of viruses in many ecosystems has been largely unexplored because characterization of viruses in biological systems historically depended on detection of observable symptoms — this was the only way to know viruses were there. However, technological advances are allowing biologists to probe the previously undetectable identities and activities of viruses in diverse ecosystems and generate a new set of basic biological questions.

> *Is there a core viral community present in each organism or ecosystem, and is it necessary to keep that system healthy, active, and productive? How heritable are viral communities? What types of virus-virus interactions take place within a given environment? How do the viruses participate in turnover of other microbes?*

These questions are as yet unanswered, but early evidence suggests that viruses and other microbes are highly coevolved at the community level and investigating the roles of viruses in these communities will yield exciting new stories. Staggeringly less is known about the roles of viruses in the higher-level biological communities within which the microbial communities are nested. Anecdotal evidence suggests that there is much to learn.

VIRUSES EXERT INDIRECT EVOLUTIONARY PRESSURES ON ECOSYSTEMS

Viruses exert profound indirect influence on the evolution of species around them. They can introduce new niche space; by targeting specific ecosystem members, they create evolutionary space for other organisms and cell types to explore. Viruses can kill off the food source of one species, or eliminate the symbiotic partner of another. Shifts in allele frequencies as a result of viral infection can have downstream effects on the environment and other organisms if those allele shifts disrupt their hosts' behavior. In the future, viruses may also serve as direct manipulators of population biology through human-controlled phage therapy.

An estimated one-quarter of fixed carbon is shunted through virus-driven processes, with viruses liberating organic carbon and other nutrients during cell death and recycling it when they themselves serve as food for other microbes.

VIRUSES ARE MAJOR DRIVERS OF GLOBAL BIOGEOCHEMICAL CYCLES

The defining element of life — carbon — is released back into the environment in staggering amounts when viruses lyse their microbial hosts. An estimated one-quarter of fixed carbon is shunted through virus-driven processes, with viruses liberating organic carbon and other nutrients during host cell death (Weitz and Wilhelm 2012). Viruses may even recycle these nutrients when consumed by certain marine zooplankton, particularly if their ingestion does not entail an infection risk (Jover *et al.* 2014). Viral lysis of the "brown tide" chrysophyte *Aureococcus anophagefferns* in coastal waters elevated dissolved iron content followed by rapid transfer of the iron to filtered particles — presumably heterotrophic bacteria (Gobler *et al*. 1997). Further, the oxygen content of one in twenty human breaths has been estimated to come from viruses stimulating oxygenic photosynthesis in the oceans (Wommack). Viruses may even affect the weather — biogenic sulfur gasses in the form of dimethyl sulfide (DMS) released during viral predation on algal blooms (Malin *et al*. 1998) oxidize into acidic particles that serve as cloud condensation nuclei in the atmosphere.

QUANTIFICATION OF VIRAL CONTRIBUTIONS TO ECOSYSTEM BIOLOGY WILL REQUIRE CONTINUED TECHNOLOGICAL AND DATA ANALYSIS ADVANCES

The connections among viruses, bacteria, and other organisms in communities are likely stable and very ancient. In marine communities, viruses contribute many genes involved in energy metabolism. Researchers can roughly estimate the viral contribution to total energy flow by using production rates for key metabolites and determining the rates of viral processes, but accurate values are still extremely difficult to obtain. Complex systems, such as the gut or soil, will have thousands of virotypes present, further complicating quantification of viral activity. Currently, methodological and knowledge gaps preclude estimating viral productivity in these systems, but the field is ripe for exploration. Further, as we increasingly recognize that global ecosystems are dependent on viruses for productivity, development of artificial systems such as bioreactors will benefit from considering viruses as drivers of productivity and stability in their models.

Two key components in the characterization of any ecosystem — establishing the identities and numbers of the players — are uniquely complicated in the viral world.

THERE IS CURRENTLY NO UNIVERSAL PHYLOGENY FOR VIRUSES

Remember that prior to 1977, the biosphere, and bacteria in particular, were classified taxonomically by appearance, metabolism, and other shared characteristics. Revolutions in molecular biology have allowed universal phylogenetic trees to be assembled based on sequence comparisons of essential housekeeping genes. Viruses, however, have been left behind.

Unlike bacteria, archaea, and eukaryota, viruses do not share a "conserved genetic core" that can be used to determine evolutionary relationships among species. There is not one single gene that all viruses share. The architecture of the three-domain tree of life was determined comparing the gene sequences for a highly conserved ribosomal subunit, but viruses do not encode their own ribosomal proteins and instead rely on hosts to supply protein-making machinery.

In the absence of a conserved molecular marker for viral phylogenetics, viruses are currently classified by host, genome type and replication strategy, and genes.

i. **Host** — In general, viruses can be separated based on the domains of life that they infect: bacteria, archaea, and eukaryota. Viruses capable of infecting members of multiple domains do exist, but have rarely been described. Viruses infecting archaea of the genera Halobacteriales are similar to Caudovirales bacteriophages and, based on morphological characteristics, have been classified among that group; future studies will reveal whether such similarities are due to convergent evolution or representative of an ancient viral lineage predating the archaea-bacteria divergence (Clokie *et al*. 2011).

ii. **Genome type and replication strategy** — Within each domain of life that they infect, however, viruses exhibit a complete spectrum of genome types and functions. Unfortunately the genomic capabilities and replication cycle are only known for a tiny fraction of viruses. These well-studied species are organized into seven "empires" via the Baltimore classification scheme (**Figure 3**).

iii. **Gene content** — Despite lack of a conserved viral gene core, specific viral families often share genome architecture and gene content, enabling evolutionary relationships within these families to be discerned.

Once a novel virus has been discovered, the International Committee on the Taxonomy of Viruses (ICTV) assigns it to a study group that decides how that virus will be classified and named. That virus is assigned a space within the existing hierarchy: family, subfamily, genus, and species; often, the new species name will reflect the host and disease it causes: *[disease] virus*. If possible, virus families are then sorted into one of seven orders, but the majority of families are not placed within this scheme. The orders are thus just groups of closely related families rather than a classification of all viruses. For example, these schemes leave out non-encapsidated viral families such as the *Hypoviridae* and *Endornaviridae*.

THE ICTV CLASSIFIES VIRUSES INTO SEVEN ORDERS:

1. **Caudovirales** — doubled-stranded DNA bacteriophages;
2. **Herpesvirales** — large, eukaryotic double-stranded DNA viruses;
3. **Ligamenvirales** — linear, double-stranded DNA archaeal viruses;
4. **Mononegavirales** — non-segmented, negative sense, single-stranded RNA plant and animal viruses;
5. **Nidovirales** — positive sense, single-stranded RNA viruses, with vertebrate hosts;
6. **Picornavirales** — small positive sense, single-stranded RNA viruses that infect a variety of plant, insect, and animal hosts;
7. **Tymovirales** — monopartite single-stranded RNA viruses that infect plants.

Biology is increasingly appreciative of the magnitude of the virosphere, its impacts on the global environment and ecology, and also the diversity of viral forms in existence.

While the ICTV classification system neatly groups most viruses into established categories, the groupings are taxonomic rather than phylogenetic because viruses of the same genome type may not be related to each other at all. Additionally, just as the recognition of a new bacterial species once required its isolation and culture, ICTV standards still require isolation of the virus itself. The vast numbers of viral sequences being generated by metagenomic techniques are currently not included in the classification system, although proposals to incorporate such sequences in the future are under discussion. Multitudes of sequences exist without taxonomic "homes," encoding a huge reservoir of new protein folds, pathogenic potential, and potential new biotechnologies. The viral universe, though it cannot yet be organized, represents a vast and exciting frontier of biology.

QUANTIFYING THE SIZE OF THE VIROSPHERE DEPENDS ON HOW SCIENTISTS DEFINE THE VIROSPHERE

Biology is increasingly appreciative of the magnitude of the virosphere, its impacts on the global environment and ecology, and also the diversity of viral forms in existence. But in the world of viruses, much is debatable — what does the "virosphere" entail? Is it merely the set of all viral entities on Earth? Does it include purely genetic elements that do not exist (at this moment) in particle form? Does it also include those genetic elements existing as reminders of ancient infections that found their way into the germline, thus propagating themselves with every reproduction of their host? Or does the term "virosphere" signify something even larger — the realm of influence of viruses on the planet? By that definition, every form of life and biological process on Earth is influenced by viral activity, past or present.

Virology as a field has traditionally focused on a very narrow part of the viral universe — that is, pathogens of humans, domesticated animals and plants, and bacteriophages. However, the field of viral ecology shows that the virosphere is enormous by almost any measure — number of viruses, diversity of viruses, and viral biomass.

i. **Numbers of viruses:** No one knows how many viruses are present on Earth, but a few estimations give a sense of scale. A "back of the menu" calculation arrives at an estimate of 10^{31} viral particles on Earth based on a 1998 estimate of 10^{30} prokaryotes on Earth (Whitman *et al*. 1998), and a general agreement among scientists that, for oceanic environments, at least, viruses typically outnumber prokaryotes by a factor of 10:1. This estimate, however, only takes into account those viral particles that can be seen and counted and does not include the many orders of magnitude of viral genomes existing within host cells, waiting to be encapsidated. The number of viruses fully or partially integrated into their host genomes is vast and significant; indeed, 8% of the human genome appears to be derived from previous viral integration events. Truly, no organism's genome operates in isolation.

ii. **Biomass and size of viruses:** Because of their sheer abundance, the mass of viruses on Earth is extraordinary. Even considering a lower bound of 10^{31} viral particles, each of which contains about 0.2 femtograms of carbon and is about 100 nanometers long, yields the viral equivalent of almost 200 million blue whales (Suttle 2005). Stretched end to end, these particles would span about 25 million light years or about 250 times the distance across our own galaxy.

iii. **Number of viral species:** Viruses are the most diverse collection of biological entities on the planet. They vary in size, shape, genome size, nucleic acid composition, replication strategy, host, genome content and function. Further, their high mutation rates and genome plasticity renders them ill suited to application of the traditional species concept. Still, one can make a few estimates. The current biodiversity on Earth is estimated at 1.7 billion species, and presuming a lower bound estimate of one virus per host yields 1.7 billion potential viral species. However, taxonomists caution that we are still not complete in our understanding of Earth's biodiversity and potential hosts; new species are reported and each one could play host to one or several viruses.

In reality, a value of one virus per host is likely a gross underestimate. Hundreds of different viruses are capable of infecting humans alone. A 1:1 ratio of viruses to different body systems (i.e. lung, liver, nervous system, etc.) or even cell types may be closer to reality. While some viruses are very host-specific, others are more promiscuous and are even capable of crossing seemingly impenetrable host barriers. West Nile virus, for example, infects mammals, birds, and mosquitos. Often, these viruses are only identified when they leave their natural host where they do not cause symptoms, further complicating estimates of their host range.

Viruses can have multiple hosts, and not all viruses infect more than one host, but an estimate of 10:1 might be a more accurate portrayal of viral diversity with respect to their hosts. It is important to remember that every time a new host is discovered, new viruses are waiting to be discovered as well — the viral universe is vast and our understanding is ever expanding.

GETTING THE MOST OUT OF VIRAL DARK MATTER?

Currently, viral dark matter sequence data is stored and curated by the scientists performing the metagenomic analyses — there is no consolidated database to which such sequence information can be submitted. Colloquium participants advocated for the creation of a viral dark matter sequence repository, one perhaps linked to existing NCBI databases, with a standardized pipeline for data submission including setting a minimum number of nucleotides as a submission cutoff. Such a database would enable comparisons across complex datasets and allow virologists to identify frequently sequenced targets for further analysis.

METAGENOMIC ANALYSES UNCOVER CONSIDERABLE VIRAL SEQUENCE DIVERSITY KNOWN AS "VIRAL DARK MATTER."

Quantifying the number of viral species is further complicated by the immense sequence diversity explored by viruses. Throughout the history of life on Earth, viruses have fluidly explored myriad genetic possibilities. While some of these possibilities lead to evolutionary dead ends (through mutations which render the virus either incapable of replicating or so virulent that its host goes extinct), as one lineage fades, new ones emerge to fill the niche.

In metagenomic analyses of all possible ecosystems on Earth, 95% of coding sequences from cellular life are similar to sequences identified previously, even if scientists have no idea what the proteins encoded by those sequences do. Conversely, 80% of coding sequences from the virosphere are dissimilar to anything sequenced before. Virologists are very far from defining the limits of viral sequence space, which is potentially vastly larger than that of cellular organisms sequence space. All of the unknown viral sequences retrieved from metagenomic sequencing are referred to as "viral dark matter." Occasionally some of these sequences will have homology to conserved unknown sequences from other databases, but often viral dark matter is not assignable to any categorized sequence groups.

Not only are viral genomic sequences diverse and unlike most things seen in cellular life, but the fully constructed genomes themselves are incredibly plastic. Viruses are not constrained by having to encode basic cellular processes on their own, as they can rely on hosts to solve many of those problems for them. With such genetic flexibility, viral lifestyles can range from pathogenic to mutualistic; currently, determining the lifestyle of a virus from sequence data alone is usually impossible. Genome plasticity and sequence diversity also gives rise to a wide variety of virus structures. Specific genomic structural features are often linked across virus families, but the virosphere is full of exceptions to the rules.

Future goals and conclusions: back to the thought question of a world without viruses.

Exploration of the viral universe indicates that a vast frontier will yield many new discoveries. It is, however, clear that it is impossible to fully understand life on Earth without considering viruses. Indeed, a world without viruses may be unimaginable. As described in this report, viruses have played — and continue to play — a multitude of roles: giving rise to the mammalian placenta and oxygen in the atmosphere, providing a mobile genetic toolkit and food for other microbes, shielding hosts from environmental and pathogenic threats, among others.

If viruses had not been playing these essential roles in biological systems, how would they have been fulfilled?

An even more profound question to consider is this: what if viruses had *never* existed on Earth? Would life have evolved quite differently? Indeed, would life have evolved at all? Answers to these questions may lie ahead as biologists begin to consider viruses as essential pieces in the great puzzle of life they seek to assemble.

An even more profound question to consider is this: what if viruses had *never* existed on Earth? Would life have evolved quite differently? Indeed, would life have evolved at all?

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