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Molecular Bases and Role of Viruses in the Human Microbiome

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<http://dx.doi.org/10.1016/j.jmb.2014.07.002>

Edited by J. L. Sonnenburg

Abstract

Viruses are dependent biological entities that interact with the genetic material of most cells on the planet, including the trillions within the human microbiome. Their tremendous diversity renders analysis of human viral communities (“viromes”) to be highly complex. Because many of the viruses in humans are bacteriophage, their dynamic interactions with their cellular hosts add greatly to the complexities observed in examining human microbial ecosystems. We are only beginning to be able to study human viral communities on a large scale, mostly as a result of recent and continued advancements in sequencing and bioinformatic technologies. Bacteriophage community diversity in humans not only is inexorably linked to the diversity of their cellular hosts but also is due to their rapid evolution, horizontal gene transfers, and intimate interactions with host nucleic acids. There are vast numbers of observed viral genotypes on many body surfaces studied, including the oral, gastrointestinal, and respiratory tracts, and even in the human bloodstream, which previously was considered a purely sterile environment. The presence of viruses in blood suggests that virome members can traverse mucosal barriers, as indeed these communities are substantially altered when mucosal defenses are weakened. Perhaps the most interesting aspect of human viral communities is the extent to which they can carry gene functions involved in the pathogenesis of their hosts, particularly antibiotic resistance. Persons in close contact with each other have been shown to share a fraction of oral virobiota, which could potentially have important implications for the spread of antibiotic resistance to healthy individuals. Because viruses can have a large impact on ecosystem dynamics through mechanisms such as the transfers of beneficial gene functions or the lysis of certain populations of cellular hosts, they may have both beneficial and detrimental roles that affect human health, including improvements in microbial resilience to disturbances, immune evasion, maintenance of physiologic processes, and altering the microbial community in ways that promote or prevent pathogen colonization.

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Introduction

Microorganisms and their viruses inhabit every surface of the human body and are recently widely recognized to be a major factor shaping human health. Cellular microbes, including bacteria, archaea, and fungi colonizing human body surfaces, outnumber human host cells by approximately 10 to 1 and play crucial roles in human health by several means including contributions to nutrition, inflammation, and immunity [1–4]. Bacteria are the most populous cellular microbes inhabiting body surfaces, and their

viruses (bacteriophage) are significantly more prevalent in humans than their eukaryotic counterparts [5,6]. There is now increased interest and research on the community of viruses (virobiota) and bacteria (microbiota) afforded by the advances in next-generation sequencing technologies and analysis tools that have improved our capabilities in the field of metagenomics. Recognizing the complexity and diversity within these dynamic viral communities has given new perspective on the human body as an ecosystem, reestablishing the role of microbes in human health and disease at the start of the 21st century.

Viruses are parasitic biologic entities that require host cells for replication. They are made up of single- or double-stranded DNA or RNA and a protein capsid, which form a structure called a virion. Some viruses may also have a lipid envelope modified from their host cell membrane. They are considered ubiquitous in that they infect nearly every type of cell (including eukaryotes, bacteria, archaea, and fungi) and are found in every ecosystem previously described [7]. Despite the large presence of phage on the planet, they were not discovered until 1915 due to their predominantly sub-microscopic existence. To this day, we remain in an age of viral discovery; most of the viruses currently being described using deep sequencing methods have not been described before and have no homologous sequences in current virus sequence databases [6,8,9]. Equally important to the next-generation sequencing are advancements in informatics allowing us to make more meaningful analyses of viromes. Tools such as the bioinformatic pipeline VIROME (Viral Informatics Resource for Metagenome Exploration) and OptItDBA (Optimized Iterative de Bruijn Graph Assembly) have greatly improved the breadth and accessibility of virome analysis by improving assembly and annotating viromes against multiple annotated sequence databases, which improves the analytic capabilities beyond the constraints of individual sequence databases [10,11]. Some molecular interactions between viruses and their hosts can be deduced from analysis of metagenomic data, which add substantially to knowledge gained from studies of laboratory-adapted host-virus model systems.

While historically many viruses in humans have been identified based on their direct pathogenic effects, we now know that there is a vast viral community in the human body that does not directly cause human disease. In fact, a large portion of the viruses identified thus far in the human virome are bacteriophage rather than eukaryotic viruses [5,6]. We will now review the current data available from studies of the human virome. Despite the current limitations in virome analysis such as the lack of available homologous sequences to identify viral community constituents, the relative dearth of information about RNA viruses in human microbial communities, and potential methodological limitations that may limit recovery of eukaryotic viruses, the continually growing number of studies focusing on viruses as members of the human microbiome provide numerous insights into the role and molecular basis of viral contributions to the human microbiome.

Bacteriophage and Bacterial Coevolution

Bacteria and their viruses have coexisted and coevolved for approximately 3–5 billion years [12]

but have been only recently shown to coexist in great abundance as members of the human microbiome. For perspective, the origin of mammals traces back to only 225 million years ago [13]; primates, to about 65 million years ago [14]; and *Homo sapiens sapiens* ancestry, to 200,000 years ago. There are an estimated 10^{31} phage on earth, based on calculations of 10^{30} bacteria on the planet [15] and approximately 10 phage that exist for every bacteria [16]. Comparatively, there are only 10^{22} – 10^{24} stars estimated to exist in the entire universe [17]. Phage are a component of virtually all molecular communities described thus far on the planet [18,19]. They are an important vehicle for exchange of genetic materials among living organisms and are likely a means by which gene functions are exchanged in the human microbiome [20–22].

Phage have been shown to be a major stimulus for evolutionary change among bacteria [16,23] and thus are dominant players in shaping the microbiota of all metazoans. They have classically been regarded as having high host specificity as an important aspect of their ecology, as many have been shown to only parasitize within a certain species and even within a subset of that species [24–28]. The increased fitness of phage with high host specificity has been largely explained by two major observations: (1) decreased efficiency for infection with broader host range [29] and (2) antagonistic pleiotropy in which an adaptation is beneficial for certain hosts but deleterious to others [30]. However, with such diversity in bacteria communities, a broader host range could be advantageous by increasing the chances of a phage encountering a suitable host cell, particularly in the setting of rare bacteria [31]. More recently, the classic view of phage host specificity has been challenged as a potential artifact from observations made on phage selected for the laboratory setting [7,32]. Instead of specificity always being advantageous over a more generalist approach, there likely exists a spectrum of viral tropism that is dependent on environmental, bacterial, and phage characteristics [33].

Phage provide great evolutionary pressure on bacteria, spurring mutations and adaptations and changing the existing gene pool. *In vitro* studies of *Pseudomonas fluorescens* and its phage have demonstrated a 10- to 100-fold increase in mutation rates over 200 bacterial generations compared to bacteria grown in the absence of phage [34,35]. Although the *in vitro* scenario of one bacteria evolving with one phage does not reflect *in vivo* evolutionary conditions, coevolution has also been shown to occur rapidly in an *in vivo* study using a “mark-recapture” strategy with *P. fluorescens* and its lytic bacteriophage [36]. The demonstration of the stimulus for evolution importantly demonstrates the significant impact that communities of phage may have as members of the human microbiome. In the setting of increased variables such as competition

among bacterial members and natural fluctuations in the host and environment, the presence of phage communities could greatly accelerate genetic evolution in human bacterial communities and potentially lead to major shifts in microbial community structures.

Phage are major sources of horizontal gene transfer among various bacterial strains, species, and even genera [37]. For example, trans-species horizontal transfer has been shown to occur with mobile toxin-carrying *Staphylococcus aureus* pathogenicity islands. *S. aureus* pathogenicity islands have been shown to transfer across species from *S. aureus* to coagulase-negative *Staphylococcus* [38] and even outside of the genus to *Listeria monocytogenes* [21]. Although there are expected fitness costs with horizontal gene transfers, they can occur without detectable losses in fitness [39]. An *in vitro* study in which DNA fragments from bacteria or phage were introduced into a specified position in the *Salmonella* chromosome showed that over 80% of insertions had no apparent impact on bacterial host fitness [39]. In the analysis of pathogens, there is evidence that horizontal gene transfers may result in conferring pathogenic traits and increased virulence to the bacterial host, such as what has been shown with increased virulence of *Salmonella typhimurium* with horizontal gene transfer via the SopE Φ phage [40]. Thus phage are powerful agents of genetic change for bacteria, enriching the genetic diversity in microbial populations and stimulating adaptation among bacteria. Given the preponderance of phage inhabiting the human microbiome, the potential for phage to transfer genes involved in pathogenicity across bacterial genera has substantial implications for human health.

Bacterial resistance mechanisms for phage

Bacteria have evolved numerous strategies for resisting their phage at every stage of attack, encouraging high phage diversity as they respond to bacterial strategies to avoid predation. The first step in phage infection involves recognition of specific host receptors, and bacteria have developed phage adsorption blocking mechanisms to escape attack by lytic phage. Some of these adsorption blocking strategies include the inhibition of phage binding to its receptor by phase variation of receptors, production of thick extracellular matrix to limit access to receptors, and alteration of receptor binding sites to escape phage attack [41]. Another common group of strategies of bacterial defenses for phage include restriction–modification defenses that involve the degradation of unmodified phage DNA. This mechanism is believed to be active in about 90% of the bacterial genomes sequenced thus far [42]. After infection with phage, a subset of bacteria actually undergo self-destructive pathways (such as

with toxin–antitoxin systems) in what seems to be an altruistic apoptosis to limit the spread of phage progeny [41,43]. There may even be ecological or epigenetic factors that affect a bacteria's resistance against phage, which may explain the recovery of a *Bacteroides caccae* population in gnotobiotic mice after experimental infection with virus-like particles (VLPs) in which there was initial bacterial decimation followed by recovery in the absence of any detectable bacterial genetic changes [44].

Another anti-phage strategy used in a large subset of bacteria are CRISPR (clustered regularly interspaced short palindromic repeats) and Cas (CRISPR-associated proteins) (referred to as CRISPR–Cas systems) in which sections of bacterial DNA that contain characteristic repeats are able to incorporate short sequences of viral DNA called “spacers” when confronted with phage. These intercepted segments of viral DNA are then able to interfere with phage genomic replication in future bacterial–phage encounters [45,46]. There are numerous repertoires of CRISPR spacers on human body surfaces, suggesting that the indigenous microbiota have CRISPR–Cas systems that are likely active in acquired resistance against phage in humans [47–49].

Bacteriophage strategies for countering bacterial resistance mechanisms

Phage continuously develop counterstrategies to evade defenses developed by their host bacteria. They have been observed undergoing point mutations [50,51], horizontal gene transfers [37,52], and genome rearrangements [53,54] in the laboratory and *in vivo* to counter bacterial defenses [55]. Numerous mutations have been shown to allow phage to escape bacterial strategies of adsorption inhibition either by being adaptive to new or modified receptor targets or by accessing receptor targets that have been protected by bacteria [55]. If phage have entered a cell with restriction–modification defenses, phage can resist these systems with mutations that prevent recognition by restriction endonucleases and thus escape cleavage [56,57]. Lysogenic phage that evolve with their host bacteria also take on similar patterns of nucleotide usage as that of their hosts and, thus, avoid using the cognate sequences of host restriction endonucleases [58]. For phage encountering bacteria that undergo abortive infection such as with toxin–antitoxin systems, phage adapt by interfering with and inactivating host cell death [54,59,60]. Recently, even phage strategies that allow for avoidance of recognition by or interference with bacterial CRISPR–Cas systems have been recognized [61,62]. Thus through adaptations and counter-adaptations, the multitudes of phage and bacteria in ecosystems throughout our world and probably in the human microbiome are constantly

shifting through evolutionary pressures imposed upon each other.

Phage provide evolutionary advantages to bacteria

The evolutionary success of phage not only stems from overcoming bacterial defenses but also includes providing selective advantages to their bacterial hosts [20]. This is particularly true for lysogenic phage that effectively are symbionts of bacteria for much of their life cycle, incorporated inside of the bacterial genome, and dependent on bacterial binary fission for its own replication [63]. There are multiple ways in which prophage (lysogenic phage incorporated into a genome) offer evolutionary advantages to their hosts through genetic diversity [64]. Phage may introduce genes that confer an evolutionary advantage in particular environmental circumstances, such that if the milieu changes, bacteria are already present with a fitness advantage. For example, in the setting of a particular nutritional milieu, two prophage within *Enterococcus faecalis* are induced to form a composite lytic phage $\phi V1/7$ that confer an advantage over other *E. faecalis* strains as seen *in vitro*, in the mouse intestine, and possibly in the human intestine [65]. Genes introduced to bacteria via prophage may offer protection of host bacteria from infection with other phage. In phage HK97 among others, superinfection exclusion has been observed, where the presence of a prophage containing a particular insertion excludes superinfection with similar phage [66]. A notorious way in which phage offer selective advantages is through the introduction of virulence factors to their hosts. Lysogenic phage are known to provide virulence factors to their host cells that can enhance survival [67–70]. Even lytic phage can select for more virulent phenotypes, as with selection for mucoid *Pseudomonas* in the presence of lytic phage $\phi 2$ [71]. In general, phage predation keeps the density of any single bacterial strain in check, which may profoundly affect the dynamics within the human microbiome, allowing for a more diverse bacterial gene pool that is better adapted to changing environmental conditions [72,73].

Phage as members of human viromes have been found to carry gene functions that are putatively involved in the pathogenesis of their host bacteria. In the human oral cavity, phage members of the virome have been found to carry genes involved in resistance to complement, degradation of immunoglobulins, adhesion to the cells lining the oropharynx, and antibiotic resistance [6,74]. In the gastrointestinal tract, phage carry substantial repertoires of antibiotic resistance, including β -lactamases, multi-drug transporters, and genes putatively involved in vancomycin resistance [75]. The fact that phage on human body surfaces carry genes putatively involved in the pathogenesis and survival of their hosts suggests

that many live in dynamic equilibrium with their cellular hosts and adds greatly to the complex dynamics occurring in the human microbiome.

Viruses Play Important Roles as Members of the Human Microbiome

Overview of human virome characteristics

Viruses, most of which are bacteriophage, exist in all surfaces of the human body [6,75]. The viromes of the human body have high levels of genetic diversity previously largely unrecognized, now being revealed with the increased detail afforded by deep sequencing methods [76,77]. The majority of the viruses inhabiting the body, as with other environments, lack homology to viruses present in existing databases [9,78]. While the identification of phage is limited by incomplete representation in these databases, the lack of available homologues does not preclude analysis of viral community dynamics [10,11]. Virome analysis of the human gut and oral cavity have revealed that membership in these communities is highly personalized [6,74,75,79]. Several studies have established that rather than a single virus predominating within a body surface's virome, there are generally numerous genotypes with no single predominant virus [6,80]. Moreover, contrary to dynamics that might be observed for simple predator/prey relationships, many phage are persistent members of the human microbiome [74,79].

There are several factors that are known to affect the membership of human viral communities. Either through personal contact or shared environmental reservoirs, individuals who live together share a significantly higher proportion of virobiota than would be expected to occur by chance, as demonstrated in oral viromes [80,81]. Host sex appears to exert an influence on virome membership as well [74]. As most of the viruses identified in that study were phage, changes in their ecology likely reflect hormonally driven fluctuations in their host bacterial communities [82–84]. While these observations suggest that characteristics observed in phage populations may reflect trends in their cellular hosts due to the complex relationships observed between viruses and their hosts in the human microbiome, relative abundances of viruses do not necessarily reflect the relative abundances of their host bacteria [6].

Viral ecology in the human gut

Many gut phage are predicted to have lysogenic lifestyles [79,85], but the majority remain uncharacterized due to lack of available homologous viral sequences. The focus of study thus far on the human

gut virome has been DNA viruses in feces, the majority of which have been bacteriophage [86]. Most of these gut viruses have been putatively classified as double-stranded DNA viruses in the *Caudovirales* order (including *Podoviridae*, *Siphoviridae*, and *Myoviridae*) or single-stranded DNA viruses in the *Microviridae* and *Inoviridae* families [78,85]. The concentration of VLPs in the human feces is estimated to be between 10^8 and 10^9 VLPs per gram [85]. While this is a high number of VLPs, the ratio of viruses to bacteria in the gut lumen (excluding the actual intestinal mucosa) is estimated to be relatively low (0.1) when compared to other environments, such as the ocean (5–10) [85,87]. In contrast to luminal viral density, the actual mucosal surfaces of the intestines are estimated to have a very high concentration of viruses and a high viral-to-bacteria ratio in comparison with the adjacent environments such as the intestinal lumen and feces [88]. The significance of the concentrated VLPs in the mucosa is likely due to mucous–phage intricate relationship discussed further in [Viromes and immunity](#) below.

Human gut viromes from healthy hosts are highly specific to individuals and fairly stable over time [75]. The stability of the gut virome was evaluated over 2.5 years during which 80% of the viral diversity was found to persist. This longitudinal study also shed light on the potential for rapid evolution of viruses on human body surfaces lending some explanation to why viromes may also be so personalized. Over the time period sampled, a lytic phage from the family *Microviridae* evolved so rapidly, it could be classified as a new species by the end of the study [86]. In addition to rapid evolution of lytic viruses, gut virome composition is closely associated with both local environment and dietary habits, as has been found in other studies. Gut viromes of individuals who experimentally consumed similar diets grew to have similar viral membership, suggesting that food could be a common reservoir from which each individual is colonized with viruses or that certain nutritional regimens alter an individual's gut virobiota based on selective pressures afforded by the nutritional milieu [75]. Interestingly, plant RNA viruses have been found in adult gut viromes, but it remains to be seen whether they represent stable members of the gut virome or are present transiently as a result of plant consumption [89]. Viruses studied in the colonization of the infant gut in the first weeks of life, however, did not resemble those from their diet (breast milk or formula), suggesting that the environment is an important initial source of viruses in infancy and likely has some influence on formed viromes as well [90].

A study by Reyes *et al.* provides the most detailed attempt to date at understanding specific dynamics of phage host populations in the human gut [44]. In this study, germ-free mice were given a 15-member

model of a human gut community. The gnotobiotic mice were then gavaged with pooled VLP extracts from human feces and then the mice microbiomes and viromes were monitored for changes in relative abundances of taxa. The simultaneous addition of diverse phage to the mouse gut did not result in a uniform decrease in abundance in susceptible bacteria but instead a variety of phage population dynamics. Two phage increased in abundance sequentially with a simultaneous drop in their bacterial host density, followed by recovery to a new steady state, while three other phage took several days to replicate to reach quantifiable abundances in the absence of any associated change in their purported host bacterial populations. In addition, mutations within a phage intergenic region were observed and appeared to confer a fitness advantage to the phage for a distinct time during the experiment. This study is thus a simplified model demonstrating some of the complex dynamic equilibrium that exists among the viruses and microbes in the gut and demonstrated that lysogenic phage provide enriched genetic diversity to the community which allows for varying fitness advantages in different environments [44].

Oral viral ecology

The human mouth provides a diverse landscape with bone, gums, soft palate, hard palate, and tongue that allows for high microbial diversity and a rich viral community [6]. Human oral viral populations consist largely of bacteriophage, with relatively few eukaryote viruses such as herpesviruses identified to date. While the gastrointestinal tract has a larger bacterial population than does the oral cavity, the mouth has been estimated to have a higher degree of mobile genetic elements (including viruses, plasmids, and transposons) than are found in the stool [91]. Many of the same trends identified in human stool viral communities also have been found for the oral virome, including that viral communities are highly personalized features of individuals [6,92]. A significant proportion of human oral viruses also are predicted to have lysogenic lifestyles, and because the virions of lysogenic viruses are stable components of the oral ecosystem, many oral viruses likely live in a dynamic equilibrium with their cellular hosts. In a recent study involving intensive sampling of human salivary viruses, the same viruses could be identified at all time points over 60 days, even when they were of low relative abundance [74]. Compared to analysis of bacterial communities using 16S rDNA, oral viruses were significantly more personalized in those subjects.

Despite the personalized nature of oral viruses, both features of the human host in which the phage resides and the human host's environment likely play a substantial role in determining human oral viral

ecology. As has previously been identified for some human bacterial communities [35,82], human oral viruses have been significantly associated with the sex of the human host [74], hypothesized as possibly a response to relative hormone levels. In the study demonstrating an association between host sex and oral viruses, a similar association could not be identified in the oral bacterial communities. This may be due to limitations of the methodological techniques for analysis of virobiota and cellular microbiota, but as demonstrated previously, abundances of phage do not necessarily correlate with their bacterial hosts in the microbiome [6,44]. It is also possible that analysis of viruses may be more sensitive than bacteria for detecting differences between human hosts.

Studies on the effect of the environment on human oral viruses have shown that the local environment contributes to determining membership of the oral virome. By examining unrelated individuals from the same households and comparing them with control subjects from different households, it was shown that persons sharing a living environment share a statistically significant higher proportion of their oral viromes compared to controls. These common viruses could reflect a sharing of viruses via direct personal contact or sharing through environmental reservoirs for viruses [81]. Alternatively, this population of shared viruses may represent similar prophage among shared bacteria. Because many oral viruses are stable members of the human oral microbiome, the sharing of viruses between household members would have value in the potential for genetic exchange between individuals.

Skin viral ecology

The skin is a major organ with important functions in protection and immunity. The viral community of the skin has been more difficult to study using metagenomic techniques than those of the gut or oral cavity due to the relative dearth of sampled biomass obtained from the skin. While there are indigenous bacterial communities inhabiting the skin [93], many of the identified viruses on the skin have been eukaryotic, including papillomaviruses, circoviruses, and polyomaviruses [94,95]. These differences in skin virobiota compared to those of the human gut and oral cavity may be due to the methodological differences used to produce the viromes, where oral and gut viromes have been enriched on density gradients and skin viromes have been deciphered from whole skin metagenomes including bacterial and human sequences. There is limited information on cutaneous phage populations; however, the fact that many skin bacteria have prophage suggests that phage are likely significant components of the cutaneous virobiota. For example, *S. aureus* and *Propionibacterium acnes* are normal cutaneous flora in humans, and each

commonly has temperate phage in their genomes. While temperate phage in *S. aureus* show high diversity over time [96], phage in *P. acnes* have been shown to be highly conserved even over a 30-year time span [97]. The relative lack of *P. acnes* phage diversity could arise from niche specialization, as *P. acnes* resides in the anaerobic follicular environment where there likely are fewer competing microbiota [98]. Interestingly, other cutaneous bacteria including species of *Streptococcus* [99] and *Corynebacterium* [100] have broad arrays of CRISPRs, suggesting that they are under constant pressure on human skin surfaces from invading viruses.

Respiratory tract viral ecology

Resident viruses in the respiratory tract have primarily been studied in the setting of cystic fibrosis (CF). The respiratory tracts in CF patients generally are heavily colonized by bacteria; thus, it is no surprise that most of the viruses characterized in the CF airways have been bacteriophage. Viromes of subjects with CF have been shown to be similar in both taxonomy and gene functions but highly disparate from the virobiota of sputum from healthy controls [80,101]. Phage genes sequenced from the CF lung appear to have adapted to the diseased lung by having increased representation of genes associated with metabolism and anaerobic processing of aromatic compounds that could improve their adaptation to biofilms and anaerobic microenvironments in the CF lung. Additionally, there are significant differences within the CF lung in viral ecology based on biogeography, indicating that the complex lung physiology impacts the local microbiota [102]. In the diseased lung, while phage adapt to the local environment, in healthy subjects, a significant proportion of respiratory tract phage likely come from the local environment. Evidence for this comes from the significant similarities observed in phage communities between a CF patient and her healthy spouse [80]. The role of the environment in the lungs is thus similar to what has been described for oral viruses [81].

Viral communities in human blood

The human bloodstream previously had been thought to be a sterile environment in healthy human subjects, but virome studies have demonstrated that they harbor substantial communities of viruses [103]. Eukaryotic viruses including Torque Teno viruses (TTVs) and SEN viruses among others have been found in the healthy human bloodstream, neither of which has been associated with pathogenesis in humans. Interestingly, both TTV and SEN viruses have also been identified in the human oral cavity [6], suggesting that these viruses may have

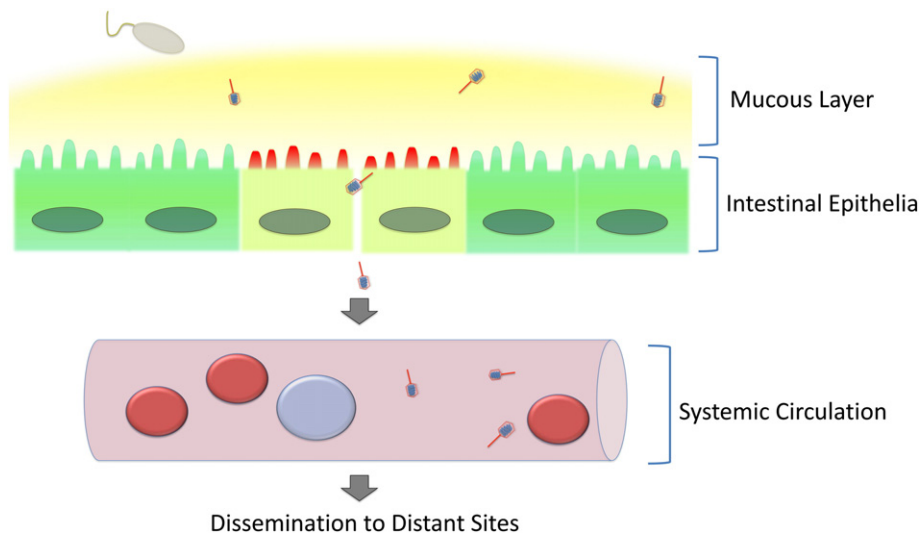


Fig. 1. Phage are able to translocate through the mucosa into blood and local tissues where they contact the immune system. Under this model, phage are able to disperse to virtually any body site and stimulate the adaptive immune system.

traversed mucosal surfaces *en route* to the human bloodstream (Fig. 1). Analysis of the viral contents of the blood in immunocompromised human subjects demonstrated the relationship between immune status and viruses, where significant immunosuppression resulted in altered viral ecology [104]. Again, TTVs were identified in the bloodstream of these subjects, which coincided with their compromised mucosal immunity. Some of these eukaryotic viruses infect hematopoietic cells, while others may enter the blood by crossing mucosa in the setting of compromised mucosal immunity. When subjects were treated with high doses of valganciclovir, their herpesviruses (generally sensitive to valganciclovir) were largely replaced by anelloviruses. Of interest, a portion of viruses identified in the blood of these immune suppressed and healthy patients were phage of the order *Caudovirales* [103]. The fact that phage have been identified in the bloodstream of both healthy and immunocompromised subjects points to blood serving as a transit for phage for access to alternate body surfaces and demonstrates that phage routinely can encounter the human immune system (Fig. 1).

Viral Communities Have the Capacity to Affect Human Health and Disease

The viral ecology of the CF lung is thought to result from the impact of the disease on the local lung environment, where genetic predisposition results in extreme alterations in indigenous microbial communities. Other disease states also probably have a genetic component to their etiology but may result in disease phenotypes driven primarily by alterations in the host microbial ecology.

Viromes and immunity

Microbiota were first observed to have a significant role in shaping host immunity, as evidenced by the fact that the absence of a microbiome results in an altered immune system in germ-free mice [105]. Phage are also proving to be a key element that helps shape innate, humoral, and cell-mediated immunity [106].

To impact the human immune system, phage must come in contact with the immune system and be able to stimulate the immune system. Phage and microbiota heavily colonize mucosal surfaces that are border regions between the outside world and the body that are both closely associated with lymphoid tissue. Free bacteriophage likely access the host immune system through translocation across mucosal surfaces such as those in the gut [107]. Similarly, lysogenic phage could come in contact with the human immune system through bacterial translocation through the mucosa and expression of phage proteins by the bacteria or through phage induction within host tissues. Once in contact with the human immune system, phage are capable of eliciting immune responses. In fact, phage stimulation of the innate immune response has been exploited for experimental anticancer therapies in mice in which tumor-specific phage activated the macrophages to secrete pro-inflammatory cytokines, attracting neutrophils to fight tumor cells [108]. It is unclear, however, if phage in their natural state on human body surfaces would stimulate the innate immune system as such. A disruption of the human mucosa or the immune system, however, allows increased quantities of phage and other viruses to gain access to the blood and tissues, possibly eliciting immune responses [104].

Bacteriophage communities have recently been shown to have a unique role in human immunity as a prominent defender of the mucous barrier from invading bacteria. The epithelial cells of mucosal barriers continuously produce mucin glycoproteins that are potential rich nutrients for microbiota [88,109,110]. Mucosa have recently been shown to be universally highly populated by phage, with a higher phage-to-bacteria ratio than would otherwise be expected [88]. To test whether this finding was important for bacterial populations, a tissue culture study was done to determine the role of lytic phage on the mucosa. The model lytic phage T4 was shown to bind to the mucus via immunoglobulin-like capsid protein interactions with glycan residues on mucinous glycoproteins. These immunoglobulin folds have been described in bacteriophage before and have been thought to have a role in interactions with carbohydrates on the surface of bacterial cells [111]. As opposed to human immune system immunoglobulin (Ig), phage Ig-like proteins diversify from targeted mutations to an exposed area of the immunoglobulin during reverse transcription (a hypervariable region), thus allowing for adaptation to diverse mucin glycoproteins and bacterial surfaces [33,111,112]. The presence of concentrated phage bound to the mucus layer results in a dramatic decrease in bacterial attachment to the mucus, resulting in significantly decreased damage to tissue culture cells [88]. This model, referred to as the “Bacteriophage Adherence to Mucus” model, is the first form of non-host-derived innate immunity described in humans and may be a key part of mucosal barrier host defenses [33].

Potential role in inflammatory bowel disease

Crohn's disease (CD) is an inflammatory disorder of the gastrointestinal tract and is thought to be linked to alterations in human gut ecology [3,113]. The disease has been studied as a state of dysbiosis for bacteria, with metagenomic studies revealing atypical and characteristic bacterial profiles in diseased intestines [114,115]. Now virobiota are also being examined for associations with the gut bacterial profiles that likely promote pathogenesis. One intriguing finding has been that there are more VLPs associated with the gut mucosa from patients with CD compared with controls [116], which suggests that shifts in gut virobiota may be a feature of CD. Additionally, there generally is lower phage diversity in CD patients compared to healthy controls [78]. While many viral communities such as those in the oral cavity generally have a high number of genotypes relatively evenly spread across the community [6], the decrease in viral diversity in CD likely is part of the dysbiosis that is pathognomonic of CD. Further studies are needed to determine whether the substantial shifts in the cellular microbial

communities are the result of changes in the virome or whether altered viral ecology just reflects changes in their cellular hosts as related to another factor such as host genotype.

Antibiotic resistance and phage

Bacteriophage are one of the major mobile genetic elements that spread antibiotic resistance genes among bacteria. Many potential human pathogens have phage that carry antibiotic resistance genes, including *Streptococcus pyogenes* [117], *Pseudomonas aeruginosa* [118], *Salmonella enterica* [119], *S. aureus* [120], and *Enterococcus* species [121] among others. Given their prominent role in horizontal gene transfers, phage can contribute to the success of their hosts in the microbiome through the dissemination of antibiotic resistance genes. Indeed, phage have been found to harbor antibiotic resistance genes in environmental water samples [122–125], soil [126], and even in some pre-antibiotic era environments [127,128]. Most importantly, phage carry antibiotic resistance in the human gut [129,130] and oral cavity [74], forming a reservoir of antibiotic resistance genes within the human microbiome [131,132]. Even if phage harboring antibiotic resistance genes are present in non-pathogenic bacteria, under antibiotic pressure, there is potential for pathogenic bacteria to acquire these phage-mediated antibiotic resistance genes [21,38].

Not only do phage communities carry antibiotic resistance genes on human body surfaces but these phage are also specifically induced in the setting of antibiotic pressure. In a mouse model, exposure to ciprofloxacin and ampicillin increased the proportion of antibiotic resistance genes found in the phageome of the gut [22]. While many antibiotic resistance genes found in these phage were putatively involved in resistance to either antibiotic, resistance to other unrelated antibiotics also increased as a result of antibiotic disturbances. In addition, interactions between phage and bacteria were noted to rise in the face of antibiotics, thus increasing the possibility for genetic exchange. Both the functionality and the transmissibility of phage resistance genes have been demonstrated by exposing antibiotic-sensitive bacteria to antibiotic-carrying phage. These sensitive bacteria then become resistant to the antibiotics themselves [22]. Thus the dynamics of phage communities are quite relevant when considering the emergence and stabilization of antibiotic resistance in the human microbiome.

Virome involvement in human host metabolism

There is strong evidence that the human microbiome is closely tied to metazoan metabolism through studies of the microbiome in conditions such as obesity and diabetes [133–135]. The role of

viruses in host metabolism has not yet been thoroughly studied, though several studies suggest that they at least have an indirect role in metabolism due to their tight relationships with their cellular hosts. Viral communities have been shown to harbor a host of genes putatively involved in the metabolic functions of their hosts in aquatic ecosystems [136,137]. However, human viral communities also carry substantial gene functions putatively involved in the metabolic functions of their hosts. For example, respiratory viruses carry gene functions involved in processing of aromatic compounds [80], oral viruses carry gene functions involved in carbohydrate and cell wall metabolism [6], and gut viruses carry gene functions putatively involved in carbohydrate and amino acid metabolism [75]. The metabolic genes present in a phageome can be altered by the host environment, as demonstrated in the adaptation of phage community metabolism to the lungs of CF patients [80].

The Role of Eukaryotic Viruses in the Human Microbiome

Although they are not believed to represent the majority of virus inhabitants of the human microbiome based on virome studies thus far, eukaryotic viruses do have a presence among human virobiota [106,138]. The skin is colonized by polyomaviruses and several different human papillomaviruses that are not believed to be associated with disease [94,139]. The respiratory tract of healthy children has been found to contain both picornaviruses and coronaviruses in the absence of symptoms, even though coronaviruses have commonly been associated with upper respiratory tract illnesses [140]. Among the bountiful phage present, the mouth also sheds human herpesviruses [141], which are also found in the human bloodstream [104] and are often shed in asymptomatic individuals. The blood also has anelloviruses in healthy individuals [142,143] and gyroviruses whose presence is related to immune suppression in the human host [144,145]. The gut also contains numerous eukaryotic viruses often sequenced in studies of children with and without diarrhea. These viruses include enteroviruses [146–148], human parechoviruses [148,149], anelloviruses [148,150], and human bocaviruses [148,151], among others [148]. There are also animal and plant viruses within the human gut [89,138] such as pepper mild mottle virus, a plant virus that has been associated with abdominal pain and fever in humans [152].

It is unclear the extent to which eukaryotic viral inhabitants of the human microbiome may contribute to human health. Many of these viruses have no known association with human disease. A recent model regarding the role of human virobiota pro-

posed is that infection with certain viruses may provide a benefit to the human host. For example, it has been hypothesized that the ubiquitous skin-colonizing β -human papillomavirus persists on human skin due to its potential to promote skin healing [153]. Likewise, human herpesviruses may benefit metazoans by priming the immune system for protection against bacteria [138,154]. As virome study methodologies improve, eukaryotic viruses may be recognized as more prominent members of the human virome. Thus the eukaryotic virome may have a tightly knit evolutionary relationship to humans, with viruses tied more closely to our genetic fitness than we currently recognize.

Limitations

While we are more advanced than the pre-omics era in our understanding of the presence of viruses in the microbiome, our knowledge remains limited. Of the data collected on viruses in the microbiome, many have used methods that might systematically exclude certain viruses. For example, most studies have been limited to DNA viruses, while the extent to which RNA viruses populate human viral communities is unclear. Plant RNA viruses have been identified in the human gut [89], yet whether their presence is transient has not been studied. Another example is the filtration techniques utilized to enrich virus particles from their cellular counterparts. These techniques necessarily exclude larger viruses that cannot pass through 0.2- μ m filters, such as the Marseille VLPs identified in human blood or *Mega-virales* that have a diameter of approximately 440 nm [155,156]. The relative lack of enveloped viruses compared to their non-enveloped counterparts in some virome studies raises some concern that methods for virome preparation are not suitable to properly preserve all viruses. Also, the underrepresentation of eukaryotic viruses in these environments not only may reflect the 10:1 ratio of bacteria to human cells but also may in part reflect an inability of virome preparatory techniques to properly preserve all viruses. Also, due to the relative lack of available homologous sequences, the majority of viruses in human viromes remain inadequately described. This has been somewhat alleviated with new bioinformatic pipelines that improve assembly and annotate reads based on the use of multiple expanded databases rather than just a single database [10,75].

There are also limitations in how to approach virus metagenome data. Most analyses of viromes are limited to the most abundant viruses present in the community, with little focus on viruses that may represent a minority of available sequence reads. As it is becoming clearer through analysis of the human microbiome, as microbial communities are disturbed,

lesser abundant microbiota can play a significant role in repopulating the community, which may have consequences for human health. Phage introduce genetic diversity into their host bacteria, and when examining these interactions on a community level, the influx of diversity may substantially alter the natural history of that community. What we do not now appreciate is whether the abundance of any individual virus or gene function carried by that virus has a large impact on its community and what might be the consequences for a rise in abundance or eradication. It may be possible that some viruses serve vital functions within their communities and that their eradication may be analogous to the removal of keystone species seen in animal predator/prey systems.

Future Directions for Analysis of Human Viromes

The virome will continue to gain importance as we recognize its intricate role as a distinct community within the human microbiome and its implications for human health. Phage are likely an important consideration for medical therapeutics. They may be an important aspect of the etiology and possibly treatment of inflammatory bowel disease and possibly irritable bowel disorder. Phage will be an important consideration in the era of personalized medicine, possibly important in determining differences in pharmacokinetics. They are likely a major contributing factor determining the efficacy of probiotics, as currently administered formulations do not generally have lasting beneficial effects on the microbiome [157]. Probiotics capable of colonizing the human gut that are resistant to common gut viruses would stand a much better chance of becoming stable members of the human gut microbiome. Because of their limited host range, bacteriophage as antibacterial therapies in humans became of limited appeal after the advent of antibiotics. However, phage are becoming more prominent contenders in medical therapies for antibiotic resistant infections [158]. Groups of phage are already important in the food industry as a targeted antibacterial approach to mitigate risk for food-borne bacterial illnesses such as listeriosis [159]. Now that we are faced with widespread antibiotic resistance, the relatively narrow host range for some phage may actually prove to be an advantage when fighting infections, as a limited host range may result in fewer disturbances to the indigenous microbiota of humans. Analysis of phage as members of the human virome has greatly added to the database of available phage genomes, and the diversity of phage in these databases can be harvested toward the goal of designing a better phage. While fighting infection is one potential use of

phage, the synthesis of recombinant phage may eventually have utility in fighting malignancies. Because humans do not generally have significant side effects from phage ingestions, their use in lieu of harmful chemotherapeutics could mitigate morbidity often incurred by conventional cancer therapies [160].

It is clear that viruses are an enormously powerful part of the intricate and dynamic human ecosystem. It is enlightening to now recognize their enormous presence on, around, and in our bodies and their potential role in health and disease as (1) individual viruses, (2) a collective community, and (3) as intertwined with the human microbiome. There is enormous potential for future work furthering our understanding about these biologic entities that are a dominant and established force in the ecology of the human microbiome.

Received 12 March 2014;

Received in revised form 30 June 2014;

Accepted 4 July 2014

Available online 11 July 2014

Keywords:

bacteriophage;
virus;
microbiome;
virome;
metagenome

Abbreviations used:

VLP, virus-like particle; CF, cystic fibrosis; TTV, Torque Teno virus; CD, Crohn's disease.

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