

Bacteriophages | Gem

The phage fought the cells, and the phage won: a satellite symposium at the ASV 2023 annual meeting

Kristin N. Parent¹**AUTHOR AFFILIATION** See affiliation list on p. 7.**ABSTRACT** This satellite symposium was focused on the molecular arms race between bacteria and their predators, the bacteriophages: who's the friend and who's the foe? This *Gem* recounts highlights of the talks and presents food for thought and additional reflections on the current state of the field.**KEYWORDS** bacteriophage, phage therapy, counter-defense, antibiotic resistance

MIGHTY, YET MYSTERIOUS

A major theme emanating from several of the talks within this satellite is how little we still know about bacteriophages both in general, as well as the intricate interplay with their hosts (Fig. 1). The microbiome is complex, and despite >100 years of research on phage biology (1, 2), there are still major gaps in our understanding. If we are to bridge this gap and robustly utilize phages for therapeutic purposes (human health, industrial, or environmental), there is much work to be done to figure out what will work routinely, as currently there is great promise and also many hurdles to overcome.

The symposium began with a field overview by Dr. Steffanie Strathdee (University of California, San Diego). Dr. Strathdee gave an account of her family's direct experience with multidrug-resistant superbugs: a personal journey to help save her husband Tom Patterson from a gastrointestinal infection that almost cost him his life. Strathdee was able to engage the scientific community in finding the right match: several bacteriophages that were specific to Tom's infection and that could reduce or eliminate his *Acinetobacter baumannii* bacterial burden. The phage cocktails he received were the result of an international "hunt" for matching bacteriophages (3). In her book, *The Perfect Predator: A Scientist's Race to Save Her Husband*, Strathdee described the challenges and opportunities inherent to phage therapy. Namely, the issues are around timing. Since phage therapy is still a "last resort" in the USA, there is a race against time when antibiotics begin to fail. In Tom's case, his phage therapy cocktails took about 3 weeks to develop with the support of three key labs across the country. Dr. Strathdee then spoke about the development of the Center for Innovative Phage Applications and Therapeutics (IPATH) and the great need for a universal, well-cataloged, and well-maintained phage library to ease the task of finding the right phage(s) for each new case. With >1,800 requests for custom phage applications since the IPATH program began in 2018, the need for a deeper understanding of phages and how to match them with multidrug-resistant hosts is pressing.

THE MIGHTY PHAGE

The next three talks focused on how phages interact with their hosts—from mechanistic and structural views of single phage:host pairs to global views of entire ecosystems.

Assistant Professor Dr. Sarah Doore (University of Florida) spoke about the complexity of phage genomes and uncovering the secrets within. By focusing solely on "model systems," the extent of the diversity and complexity of phage biology can be missed. For

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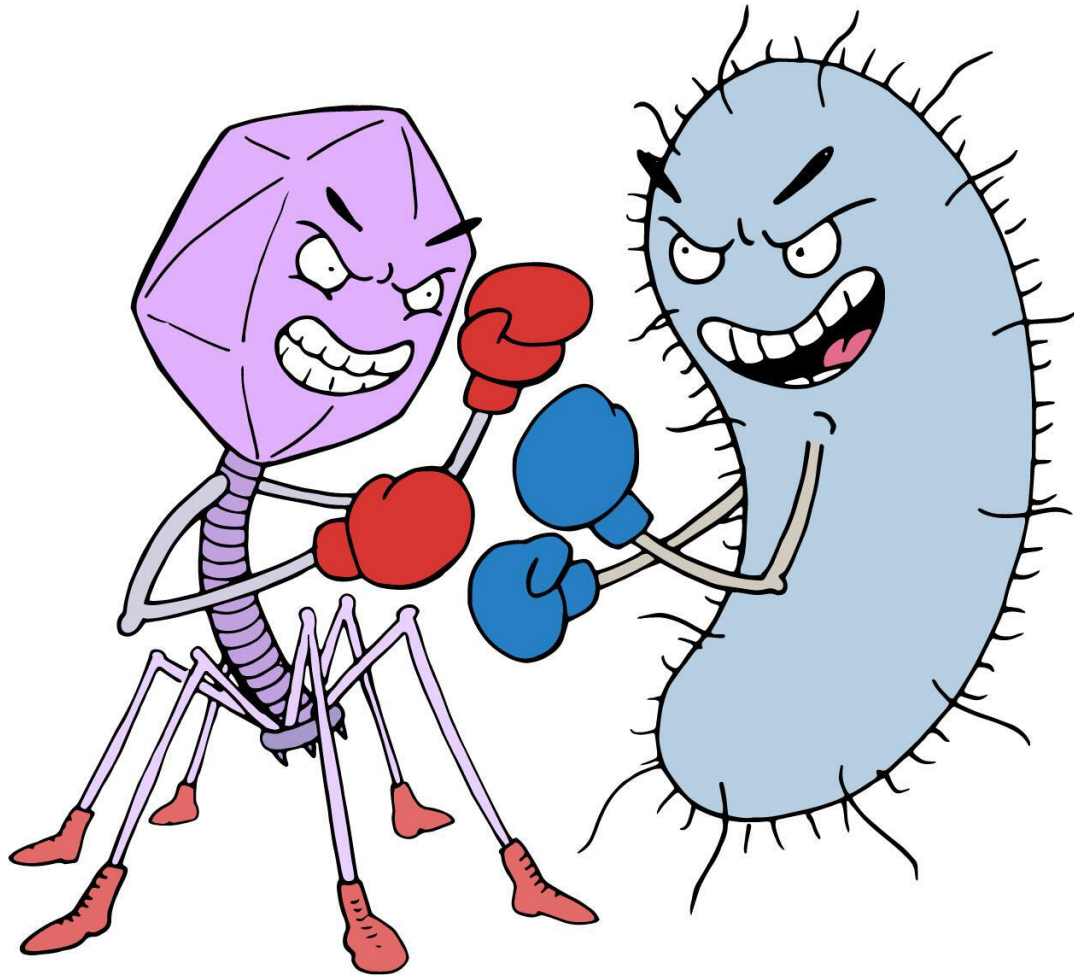


FIG 1 Bacteria and bacteriophages face off and fight each other in an ongoing molecular arms race. Image credit: Patrick Lane, Sceyence Studios.

example, a large number of *Shigella* phages isolated from the environment have a common size, morphology, and genome architecture to one another, yet the features of these *Shigella* phages are unlike the well-known *Escherichia coli* phages (4), with very little crossover for infection despite their hosts being similar. For these so-called Mooglyphages, there are a striking number of genes related to the process of translation, with 25–27 genes dedicated to encoding tRNAs (5). Why would phages devote so much of their coding capacity toward this basic process? It is not simply a “bigger is better” phenomenon as Mooglevirus genomes tend to span 80–95 kbp, yet their significantly larger, but more distantly related cousin, phage T4 (~145 kbp) typically only encodes approximately four tRNAs. Dr. Doore then took a deeper look into how Moogleviruses infect their hosts and how sugar is not always nice—the lipopolysaccharide (LPS) candy coating on *Shigella* is complex, and if phage therapy is really going to take off, we need a deeper understanding on how phage tail architecture(s) interact with LPS—a structure that can evolve rapidly during the arms race. The LPS molecule can be broken down into four different regions, the O-antigen, the outer core, the inner core, and the Lipid A molecule; see Fig. 2 and Discussion in Bohm et al. (6) for a schematic of the LPS molecule, a schematic of the biosynthetic pathway, and details of LPS conservation across enteric bacteria. The distal end (O-antigen) is highly varied, and considering the genus *Shigella*, it is prevalent only in *Shigella flexneri* species. As we approach the cell surface, the conservation increases with the outer core conserved within species, whereas the inner core and Lipid A are conserved between species. Since LPS is such a complex molecule, lots of experimental evidence and gene knockouts need to be created in order to glean the full

picture. Even then, attachment and genome ejection into live cells can be highly variable (unpublished results), making these studies difficult and time-intensive. However, if we are able to dissect the rules of attachment, we will be better armed to fight the battle against superbugs, by using this knowledge to narrow down potential candidates for the development of therapeutic cocktails and focusing on entry mechanisms that are resistant to constantly evolving serotypes.

Diversity of known phages, surface interactions, and the mysteries hidden within the genome have serious implications for successful phage therapy. Dr. Graham Hatfull (University of Pittsburgh) talked about his experience creating the Science Education Alliance-Phage Hunters Advancing Genomics and Evolutionary Science (SEA-PHAGES) program (7) and surprises from mycobacteriophages. With >40,000 students participating, >23,000 isolated and archived phages, and >4,400 sequenced and annotated genomes, this program has contributed the largest single body of data on phage diversity in history. There is a fantastic diversity of phages. But as Dr. Hatfull points out, the unknown expanse of the phage universe is an inhibitor for making real forward progress in developing them as therapeutics. In a case study of persistent multidrug-resistant *Mycobacterium* infections after a bi-lateral lung transplant in two cystic fibrosis patients, Hatfull was faced with the daunting problem that the majority of mycobacteriophages are lysogenic or temperate (meaning they do not immediately kill their hosts), and despite the wealth of information gleaned from SEA-PHAGES data, over 70% of their annotated genes still have unknown function. After a massive screening of thousands of phage stocks, three candidate phages would likely be useful for a cocktail. Hatfull made use of his creative methods for engineering phage genomes (8, 9), safe administration was then made possible by rendering the phages lytic, and ultimately, the infection was significantly reduced for one patient (10). Hatfull then went on to talk about 20 other case studies and the efficacy of using phages to treat bacterial infections. The take-home message is, in our current state, “sometimes it works, sometimes it doesn’t and we don’t know why.” But, when it does work, it is encouraging enough that we should keep trying as, for some patients, this is literally the only option. One silver lining is that studying groups of phages can elucidate information that can help wield this mighty natural power by exploiting unique properties. Hatfull described how, sometimes, sugar can indeed be very nice: how glycosylation of phage capsids and tails in the mycobacteriophage family can actually boost the immune response by shielding the particles to help fight against *Mycobacterium* infections (11). Currently, phage therapy is highly personalized, and we are mostly guessing at administration routes, doses, and duration with little empirical evidence to drive the therapeutic plan. However, combining learned phenomena with the ability to engineer modified phages could really be an advantage in the coming years.

We have heard about the intricacies of the molecular “match maker”—finding the exact right binding partners between phage and host. Dr. Forest Rohwer’s (San Diego State University) research takes a broader view and focuses on the abundance, diversity, and trends that cause large-scale shifts in entire ecosystems. Finishing out the first session, he talked about strategies of “piggyback the winner” and how phages regulate the lytic versus lysogenic switch in different group scenarios. For example, in marine ecosystems, increasing microbial density tends to harbor more viruses overall but drives lysogenic life cycles instead of lytic ones. Defense mechanisms such as superinfection exclusion, serotype conversion (the process by which bacterial cell surface features are modified, based on phage-encoded enzymes), and metabolic pathway changes all can drive this lifestyle swap in a dynamic manner that is a consequence of a highly complex web of microbial interactions (12). A comprehensive look at phage:host interplay in coral reefs (13) and cystic fibrosis patients (14) tells us it comes down to metabolism. Phages are highly evolved puppet masters that monitor their hosts’ redox potential and energetic flux in order to do their bidding. More ATP is needed to produce new progeny; therefore, low (ATP) and high (NADPH) are drivers of lysogeny. However, we also need to look at spatial distribution as well, which can add increasing complexity

when contemplating treatments (15). If we are to look at the health and shifting trends of any complex system, we need to use what we learn about both localization and energy management from these diverse ecosystems in order to accurately predict outcomes. Using diseased coral reefs as a test case, “dynamic viralization” (or shifting a community to have a higher virus-to-microbe ratio) can reverse the process of “microbialization” [which is when an ecosystem shifts toward microbial overgrowth (16)] by increasing oxygen levels using coral reef arks (platforms seeded with corals that float a few meters above the ocean floor) designed to monitor and potentially rebuild ecosystems (17). Such predictions will be especially important as we start employing phages as therapeutics to treat human diseases such as cystic fibrosis (18). In order to deconstruct what is happening during a human health intervention, we need “BACI” (Before/After Controlled Impact) and collect data on each patient as they move through time. As Dr. Rohwer points out, dissecting the patients to discern what is going on during treatments is “often really frowned upon.” But, tracking microbiology and all the “omes” (transcriptomes, metagenomes, viromes, metabolomes, etc.) and temporally analyzing these data from collected patient samples can then be used to actively inform treatment. For example, manipulation of the system by adding electron acceptors to increase oxygen can activate prophages as part of the cystic fibrosis rapid response (19). Entire ecosystems and the history of individual human health are highly complex and take holistic views of the entire microbiome to fully understand.

THE CELLS FIGHT BACK

The last three talks in the symposium focused on bacterial anti-phage defense systems and how bacteria can survive even under constant duress from predators. Prokaryotic immune systems represent a new area of research, and new emerging trends are discussed. One key secret to evading host predation is a type of acquired immunity called Clustered Regularly Interspaced Short Palindromic Repeats–CRISPR-associated proteins (CRISPR-Cas). In these systems, DNA fragments from previous phage infections help the bacteria recognize the incoming predator and stop the infection in its tracks. There are three stages of CRISPR-Cas immunity: adaptation (spacer acquisition), expression and processing, and interference (nucleotide cleavage). In this second session, we heard about several ways cells use CRISPR-Cas to fight phages and how the phages can bypass CRISPR-Cas immunity at different stages (20).

Starting off the second session, Dr. Sylvain Moineau (Université Laval, Canada) talked about how to achieve the exact opposite goal as discussed in the first session: the need to thwart phages in industrial settings to protect the “good” microbes (21). This is most notable in fermentation processes, saving the dairy industry from harmful phage predation against lactic acid bacteria (22). In sharp contrast to the high prevalence of temperate *Mycobacterium* and marine phages discussed previously, a vast majority of known *Streptococcus thermophilus* phages are virulent (or obligately lytic), making these phages a costly threat to the yogurt and cheese industry. For most countries, the only way to reduce the phage threat is through creating resistant bacterial cultures by non-genetically modified organism methods. For example, exploiting natural anti-phage systems makes CRISPR-Cas-based immunity a great option (21). In contrast to the enormous diversity seen in *Mycobacterium* phages, there is a paucity of phage diversity infecting *S. thermophilus*—with—with only five genera (so far), making CRISPR-Cas systems particularly useful when targeting conserved phage genes. In support of its importance in this food-grade bacterial species, 100% of the publicly available 87 complete genomes of *S. thermophilus* display CRISPR-Cas systems (specifically Type II-A) (23). CRISPR-Cas systems can also work in tandem with other methods of phage resistance mechanisms, such as the innate immunity gained by restriction-modification systems (24) to increase resistance against phages. By treating cells with a “whole truck of phages,” resistant colonies can be isolated and sequenced. Unlike *E. coli* or other enteric pathogens, receptor mutants were not recovered when facing extinction by *S. thermophilus* phages. Instead, only cells that had acquired immunity through CRISPR

were identified (25). This effect can be stacked—challenging *S. thermophilus* with diverse phages can lead to adaptive immunity against multiple types of phages leading to the idea that the more spacers one has acquired, the greater the immunity the cells harbor. Yet, in a marvelous display of evolution in action, phages can still strike back and escape CRISPR immunity. Single-nucleotide substitutions can thwart the recognition by spacers quite effectively (26). In addition to the single-nucleotide escape mutants, CRISPR-Cas evasion can be achieved with phage-encoded anti-CRISPR proteins (ACRs) (27). Moineau talked about various ways *S. thermophilus* virulent phage ACRs inactivate CRISPR-Cas immunity, including broad and narrow inhibitors and ones that can have different modes of action such as bypassing both interference and adaptation stages (28–30). Much like Rohwer stressed the importance of monitoring phage:host interplay with time in clinical settings, Moineau has also monitored the arms race between phage and host across an astonishing 20-year period using another dairy bacterium, namely, *Lactococcus* (31). This work has generated a new library of phages and has guided the industrial partner in a cheddar cheese factory to add or remove strains to adapt their starter cultures, showing a very practical application to understanding the dynamics of other types of immunity and evasion. Similar to what Strathdee described for IPATH, there is a need for a well-characterized and maintained library of strains to benefit industry as well. Since 2003, Dr. Moineau has been the curator of a phage collection (www.phage.ulaval.ca) established in 1982 at the Université Laval by Dr. Hans-W. Ackermann and is maintaining an amazing wealth of knowledge and strains.

Next, Dr. Asma Hatoum-Aslan (University of Illinois, Urbana-Champaign) continued to discuss CRISPR-Cas systems (32), focusing on the Type III-A system, in a “not so good” microbe, *Staphylococcus epidermidis*, which is a commensal bacterium prevalent on skin that has the potential to cause antibiotic-resistant infections. The Type III-A systems are widespread in nature and target the RNA of phages, using a multiprotein effector complex. Hatoum-Aslan has shown that the Type III system in *S. epidermidis* recruits several cellular, “housekeeping” enzymes to achieve phage defense—PNPase, RNase J, and RNase R (33–35). In a series of detailed biochemical assays, Hatoum-Aslan was able to demonstrate that these nucleases play essential roles in multiple steps of the CRISPR immunity pathway, including CRISPR RNA processing and interference. The takeaway message of this work is that this type of CRISPR-Cas system is not self-contained: it is unable to work efficiently on its own and has evolved to co-opt cellular resources and machinery in order to maintain robust immunity. So how robust is this immunity? The challenge from *S. epidermidis* phages usually results in efficient killing, where 100% of phages are completely wiped out, owing in part to the fact that Type III systems are protospacer adjacent motif independent (36). However, Hatoum-Aslan’s group observed that a small handful of myophages have the capacity to bypass *S. epidermidis* Type III-A immunity (unpublished results). About half of the talk focused on their efforts to determine the gene responsible for this anti-CRISPR activity and characterize the mechanism of action. Their work has uncovered a new mode of action for an ACR. There is much excitement to come in this area.

Dr. Peter Fineran (University of Otago, New Zealand) finishes the satellite symposium with a flourish talking about a multitude of resistance mechanisms. One critical point made by Fineran is that to make sense of phage:host interactions, we need the tools to detect phage defense systems in the growing number of bacterial genomes. Together with Dr. Simon Jackson, they have recently created **Prokaryotic Antiviral Defense Locator** (“PADLOC”), which is a platform that can predict defense systems in bacterial genomes (37, 38). Given the selective pressure of CRISPR, it is perhaps unsurprising that phages have adapted a wide variety of anti-CRISPR escape strategies (20). Some exotic methods uncovered by the Fineran group include the jumbo phages, which can create bizarre nucleus-like structures to protect the DNA during infection but can still be vulnerable to attack against RNA as this is exported from the nucleus-like structures throughout the lifecycle (39). The majority of his talk focused on anti-CRISPRs, including RNA-based anti-CRISPRs and anti-CRISPR regulation. Some phages contain solitary repeat units

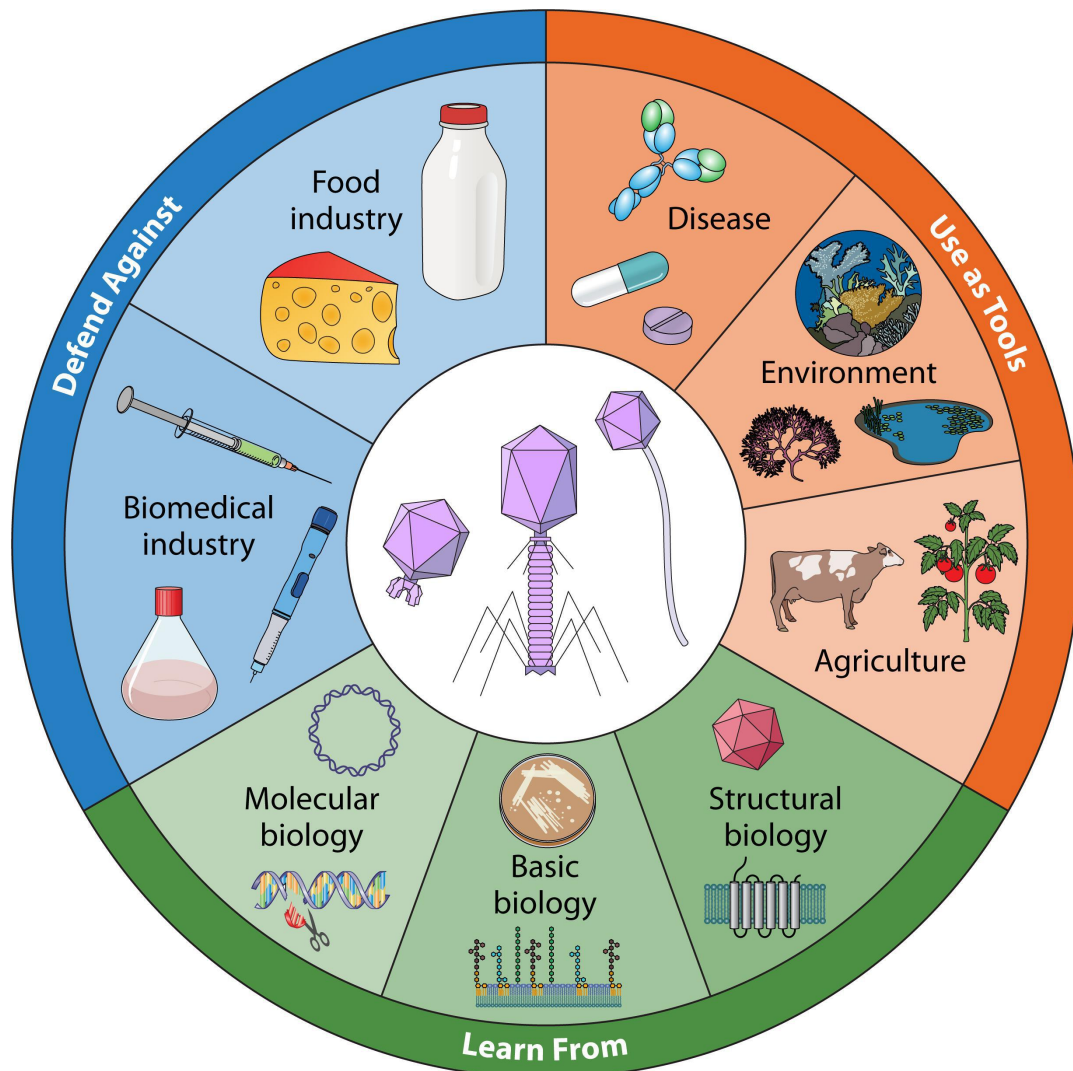


FIG 2 Potential avenues for the development of phage therapeutics, anti-phage defense strategies, and basic research using phage to discover novel mechanisms and biology. Phages' natural ability for infection is often harnessed as a way to decrease specific bacterial burdens. Disease prevention includes arming cocktails of phage as killers against multidrug-resistant bacteria and using phage to help boost the immune system. Use of phage as environmental tools includes restoration of healthy microbial balance in complex microbiomes. Agricultural applications of phages can help mitigate bacterial disease in livestock and crops. There are also instances when we want to keep the phages at bay. The dairy industry relies on healthy bacterial cultures, so defending against phage is paramount. Likewise, the biomedical industry often uses bacteria to produce any number of products, and phage infections would jeopardize production of life-saving medicines, such as insulin. Lastly, learning about phage: host interactions can benefit science as a whole including guiding modern molecular biology and gene-editing strategies, gaining a fundamental understanding of biology such as cell surface properties, and understanding the basics of protein structure and complex assemblies that could downstream lead to structure-guided engineering of more effective phage particles. Of course, there are other potential areas of importance, but this schematic focuses on the topics covered at this symposium. Image credit: Patrick Lane, Sceyence Studios.

(SRUs) that are localized away from CRISPR-Cas-associated genes but are found in many phage genomes (40). By testing these SRUs for anti-CRISPR activity, the Fineran group, in collaboration with researchers in Copenhagen, discovered short-expressed non-coding RNAs that inhibit CRISPR-Cas activity, so-called Racrs, which are RNA-based anti-CRISPRs (41). It appears that Racrs are prolific in mobile genetic elements and will likely emerge as a widespread mechanism for phage defense against pressure from CRISPR inhibition. The next theme is controlling ACR regulation—how to time it so the cell is protected but not wasteful and in a way that products will not build toxicity. It is clear that we would be

wise to stay tuned to learn more about exciting anti-CRISPR systems and their underlying mechanisms from the Fineran group.

Identifying the plethora of defenses both bacteria and phages have in their arsenals will drastically help us in the future as phage therapy becomes more critical in industrial, environmental, and human health contexts.

ADDITIONAL POINTS FOR REFLECTION

The overarching message is simple: phage therapy is promising and can have a wide reach (Fig. 2) but is currently underdeveloped. Finding the right phage is a “needle in a haystack” with no systematic database yet fully in place. If we gain a molecular mechanistic understanding of phage interplay with their hosts, we can crack the code and find the right match or even design the right phages to treat patients more effectively and rapidly. Treatments need to be monitored with time and strategies revisited as the landscape evolves. Other battles we have to face are cultural. Dr. Strathdee talked about the perception of “older, Soviet science versus modern Western medicine” and the impact this is having on buy-in, thus currently negatively hindering policy and controlled clinical trials. Another grim reflection is that as mighty as phages are, if we do not have a good understanding of their biology and their administration in public health, we may face additional resistance problems, much like that with antibiotics, and then what will we do?

We can begin to solve these problems with more resources. Funding basic research on phage biology to reduce the knowledge gap is the first step. We need cooperation for regulatory approval from the Food and Drug Administration buy-in to support clinical trials and reduce red tape when life-saving measures are needed. Clinical trials using phage as therapeutics against opportunistic pathogens are slowly taking off, including the recent US-based trials on treating conditions such as cystic fibrosis (NCT05453578) and enteric pathogens in Crohn’s disease (NCT03808103). However, more trials are sorely needed. We also need better information flow between medical professionals and basic research teams. When compassionate cases arise, hopefully, we can evolve from individuals only reaching out in time of need to a better standing network of colleagues, which ultimately can bridge often siloed clinical and academic groups to speed up the pace for phage therapy in dire cases. Phage therapy is seeing a “renaissance,” but there is much work to do before we can fully embrace this technology (42). Given the “Red Queen hypothesis” where evolution is akin to species “running as fast as they can to stay in the same place,” phages and bacteria will continue their never-ending war. But, once we get a handle on the metagenomic dark matter and key biochemical phenomena, by uncovering new mechanisms and understanding the diversity of phage resistance, we can start intervening. Hopefully, then we can win an increasing number of battles by guiding evolution toward even more productive therapeutics.

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