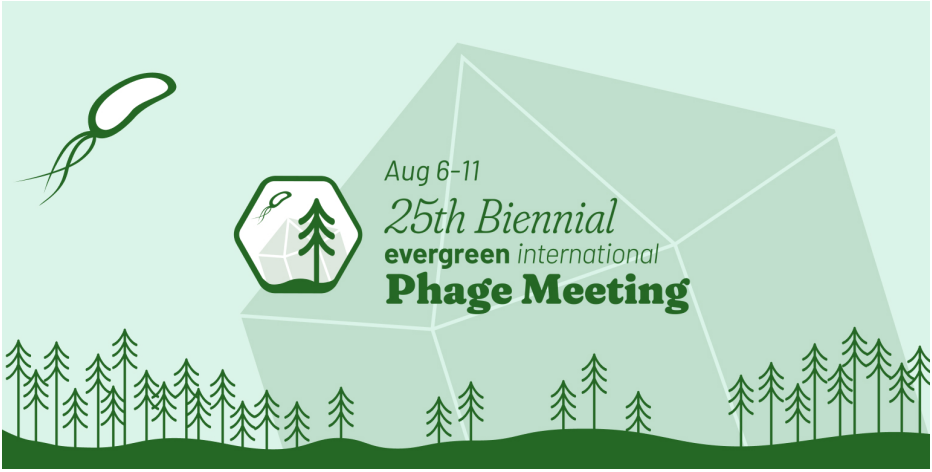
25th Biennial Evergreen International Phage Meeting



August 6-11, 2023

The Evergreen State College, Olympia, WA

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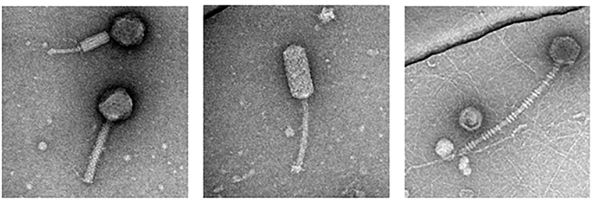

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Tailored **A**ntibacterials and **I**nnovative
Laboratories for phage (**Φ**) **R**esearch

Baylor
College of
Medicine

25th Biennial Evergreen International Phage Meeting

The Evergreen State College, Olympia, WA

Coordinating Committee & Organizational Staff: Elizabeth Kutter, Chair; Ria Kaelin, Jan Zheng, Jessica Sacher

Science Committee: Evelien Adriaenssens, Jeremy Barr, Sandra Morales, Sabrina Green, Steve Abedon, Hany Anany, Ria Kaelin, Jessica Sacher

Workshop Presenters: Evelien Adriaenssens, Ryan Cook, Claire Elek, Andrea Telatin, Justin Clark, Ramy Aziz, Rebecca Wattam, Tobi Nagel, Fran Hodges, Jason Gill

Meeting Goals Building on the original Cold Spring Harbor phage meetings, the Evergreen meeting's primary goal is to provide an opportunity for members of the ever-broadening phage community from various backgrounds, ages and places to build strong working relationships and share skills and ideas, leading to new insights and collaborations and, in recent years, helping spur on many kinds of applications. People are encouraged to present work that is still in progress. Posters are strongly encouraged, both as independent presentations with their own abstracts (grouped by general topic) and as a way of letting participants more closely examine the detailed data of those giving talks. They remain up throughout the meeting, rather than having specific presentation times for individual groups of posters.

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Special thanks to the following organizations for their financial help in supporting young scholars and participants from underdeveloped countries; please look at their postings at the end of the abstract book: TAILOR, JAFRAL, Phase Genomics, PHAGE Therapy, Applications, and Research, Phages for Global Health, Rime Bioinformatics, DPI Drugs and Drug Candidates, Vésale Bioscience, Phage Directory, Phagebiotics Research Foundation

Session 1: Plenary talks

Session 2: Ecology & Informatics I

Session 3: Phage defense

Session 4: Phage therapy I

Session 5: Phage therapy II

Session 6: Understanding & improving phage therapy

Session 7: Phage and the immune system

Session 8: Molecular phage biology I

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Session 11: Phage in food and agriculture

Session 12: Molecular phage biology II

Poster Session

Session 1: Plenary talks

bacteriophages endolysins antibiotic resistance synthetic biology genetic engineering phage-based antimicrobials

Bacteriophages by design: tiny killers and smart detectives

Martin J. Loessner [1*]

1. Institute for Food, Nutrition and Health, ETH Zurich, Switzerland

Antibiotic resistance represents a great challenge for health care worldwide, and novel strategies to control infection by drug-resistant bacteria are urgently needed. While the natural enemies of bacteria, bacteriophages, offer great host specificity and killing activity, their therapeutic potential is naturally limited by narrow host-ranges, insufficient antimicrobial activity, lysogeny, and rapid emergence of resistance. However, synthetic biology and genetic engineering of phage genomes can overcome these limitations and offers new possibilities for the design of smart and effective phage-based antimicrobials.

We use a synthetic phage engineering approach, based on in-vitro DNA assembly and subsequent reactivation (rebooting) of synthetic phage genomes within suitable host cells. To enable efficient rebooting of phage genomes in Gram-positive bacteria, we developed a bacterial L-form based platform, providing less stringent surrogate hosts for phage amplification. To enhance recombination-based engineering of very large phage genomes unsuitable for synthetic fragment assembly, CRISPR-Cas based counterselection systems were established in various phage hosts. Using these platforms, we (i) converted temperate phages to virulent ones, (ii) produced phages carrying a broad variety of additional payload genes for expression in the infected host, (iii) created phages showing extended killing of completely unrelated bacteria by phage-encoded cross-acting antimicrobials, (iv) designed transducing but non-replicating killer phage, (v) broadened phage host ranges by structure-guided design of receptor binding proteins, and (vi) provided a corresponding arsenal of reporter phages for companion diagnostics prior to administration in clinical trials.

Besides using bacteriophages, another very successful approach is to harness the bacteriolytic endolysins. Here, we have made significant progress by not only optimizing enzyme activity and in vivo half-life by domain shuffling and fusion to non-phage sequence, but also targeted modification of the enzymes for fine-tuned application in serum and blood, tissue, and intracellular environments.

phage taxonomy

What's going on with phage taxonomy and is it really necessary?

Evelien M. Adriaenssens [1*]

1. Quadram Institute Bioscience

In this talk, I will discuss the latest updates in phage taxonomy by the International Committee on Taxonomy of Viruses (ICTV). In the last two years, phage taxonomy has undergone a major update with the abolishment of the morphology-based families Myoviridae, Podoviridae and Siphoviridae. All phages in these families have been reassigned to new genome-based families or are currently grouped into the class *Caudoviricetes* which has replaced the order Caudovirales.

I'll explain why this update was necessary, the history behind it, and how it will help your analyses, whether you work with novel phage isolates or at the viral community level.

Session 2: Ecology & Informatics I

Metagenomics phage profiling

Phage-inclusive profiling of human gut microbiomes with Phanta

Yishay Pinto [1*], Meenakshi Chakraborty [2], Navami Jain [3], Ami S Bhatt [4]

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2. Department of Medicine, Divisions of Hematology and Blood & Marrow Transplantation, Stanford University, Stanford, CA, USA

Due to technical limitations, most gut microbiome studies have focused on prokaryotes, overlooking viruses. Phanta, a virome-inclusive gut microbiome profiling tool, overcomes limitations of assembly-based viral profiling methods by using customized k-mer-based classification tools and incorporating recently published catalogs of gut viral genomes. Phanta's optimizations consider the small genome size of viruses, sequence homology with prokaryotes, and interactions with other gut microbes. Extensive testing of Phanta on simulated data demonstrates that it quickly and accurately quantifies prokaryotes and viruses. When applied to 245 fecal metagenomes from healthy adults, Phanta identifies ~200 viral species per sample, ~5x more than standard assembly-based methods. We observe a ~2:1 ratio between DNA viruses and bacteria, with higher inter-individual variability of the gut virome compared to the gut bacteriome. In another cohort, we observe that Phanta performs equally well on bulk vs. virus-enriched metagenomes, making it possible to study prokaryotes and viruses in a single experiment, with a single analysis.

phage genomics | machine learning | bioinformatics

Phage genomes - taming the unrecognisable, uncategorisable and uncomparable.

Thomas Sicheritz-Pontén [1*], Martha Clokie [2]

1. Globe Institute, University of Copenhagen, Denmark
2. Leicester Centre for Phage Research, UK

Phages were first isolated and used as therapeutics around a century ago. They were used independently of any genetic knowledge and ultimately abandoned due to their complexity and because antibiotics were simpler to develop. This complexity is now what is needed to be channelled into medicine to produce much needed antimicrobials to treat antibiotic resistant bacteria. However, using phages therapeutically is challenging on many levels. Unlike the situation a century ago, investigating phages in the 2020s can exploit the wealth of genetic information encoded in their genomes. As phage genomes are remarkably diverse with hardly any conserved sequences, bioinformatics analysis is highly challenging where traditional bioinformatics tools compare genomes and genes based on DNA or protein sequence similarity. The high degree of genetic variability can make it difficult to accurately annotate phage genomes and predict the functions of their encoded proteins - the majority of phage genes have no homologs in existing databases, complicating the understanding of phage biology. The vast genetic diversity also hinders genome-wide comparisons of phage genomes, where typically less than 25% of genes within individual phage genomes have sequence similarities, and generally only within closely related groups.

To address these issues, we are developing new methods using feature based machine learning (based on the observation that proteins with similar functions can share features, despite being far apart in sequence space), ecology analysis (based on our observation that prediction of transcriptional take-over strategies allows the identification of ‘Phage functional types’) and social network graph theory (based on the observation that genomic networks display patterns of interactions that are similar between phages targeting different bacterial hosts). These methods will aid clinical, agricultural, and industrial phage therapy by identifying similar phages and predicting content similarity without relying on sequence similarity.

Phage Plasmids Conjugation Pilus Tectivirus

Phage DISCOvery in technicolor reveals diverse and abundant phage populations exploiting conjugative plasmids

Siân V. Owen [1*], Natalia Quinones-Olvera [1], Lucy M. McCully [1], Maximillian G. Marin [1], Eleanor A. Rand [1], Alice C. Fan [2], Oluremi J. Martins Dosumu [3], Kay Paul [3], Cleotilde E. Sanchez Castaño [3], Rachel Petherbridge [1], Jillian S. Paull [4], Michael Baym [1]

1. Harvard Medical School
2. Boston University
3. Roxbury Community College
4. Broad Institute of MIT and Harvard

Plasmid-dependent phages are bacterial viruses which unusually, instead of depending on chromosomally encoded receptors, use receptors encoded on conjugative plasmids. This gives them exceptionally broad host ranges, and movement of a conjugative plasmid through a bacterial community can rapidly create large pools of phage-susceptible bacteria. Plasmid-dependent phages also belong to unusual groups of non-tailed phages including Tectiviridae, Inoviridae and Fiersviridae. Despite their interesting biology and potentially profound effect on the ecology and evolution of horizontal gene transfer, technical challenges have impeded their study. We have developed a simple fluorescence-based method, phage DisCo (Discovery by Coculture) that allows us to systematically isolate phages that infect bacteria carrying specific conjugative plasmids.

In this talk, I will describe how we applied this method to isolate an unprecedented collection of novel plasmid-dependent phages. We discover that rather than being rare biological oddities, these bacteriophages are common and hugely under-sampled: the diversity we uncovered in Boston (USA) vastly expands the known global diversity, encompassing several putative novel species. Furthermore we find these phages are surprisingly absent from metagenomic datasets, hinting at the failure of metaviromics to capture them. Using high-throughput phenotypic profiling we find evidence for host range restriction amongst certain phage isolates, and we are actively exploring how both plasmids and bacteria defend themselves against plasmid-dependent phages.

The discovery of these diverse phage populations has important implications for horizontal gene transfer, and likely represents an unexplored, and potentially exploitable, force shaping the ecology and evolution of antibiotic resistance in bacteria.

meta-omics | stable isotope probing | biogeochemistry

Soil virus forensics: throwing the kitchen sink at viruses in soils to characterize their activities.

Gareth Trubl [1*], Grant Gogul [2], Ella Sieradzki [3], G. Michael Allen [1], Jeffrey A. Kimbrel [1], Dariia Vyshenska [4], Robert Riley [4], Graeme W. Nicol [3], Christina Hazard [3], Joanne B. Emerson [2], Simon Roux [4], Rex Malmstrom [4], Emiley Eloe-Fadrosh [4], Steven J. Blazewicz [1], Jennifer Pett-Ridge [1]

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3. École Centrale de Lyon, France
4. Joint Genome Institute, Berkeley, CA

Soils contain between 1500–2400 gigatons of carbon and the fate of this carbon is unknown as Earth's climate changes. One of the largest sources of uncertainty is predicting and understanding microbial biogeochemistry. Viruses that infect microbes can be major players in microbial biogeochemistry via top-down controls by lysing microbes and releasing their necromass, and bottom-up controls by modulating microbial metabolism. Research on the diversity and functional capabilities of soil viruses is expanding near-exponentially, however, current methods are fragmented, limiting our ability to make connections important to soil and global ecology. We hypothesize that virus-microbe interactions and microbial ecophysiology fundamentally shape soil carbon persistence and we focus on soil moisture as a 'master controller' of viral and microbial activity. We simulated a precipitation event using heavy water (H218O), treated half of the samples with a phosphorus addition (It has also been shown that phosphorus concentration can affect the number of virus particles produced during an lytic infection), and sampled over a month (T0, 1, 2, and 3 weeks) with stable isotope probing targeted metagenomics (to identify active organisms and proviruses) and viromics (to identify active virions), combined with bulk metatranscriptomics (to identify RNA viruses and capture gene expression) and environmental DNA (eDNA) surveys (to characterize the origin and persistence). A bird's-eye view of the data revealed in the dry soils eDNA and integrated RNA viruses are very abundant, but then one week after wet-up, we see a surge of microbial and integrated DNA virus activity coinciding with a large production of carbon dioxide (CO2) and a decrease in eDNA and integrated RNA viruses. The second week after wet-up, we see another surge in DNA virus activity but in virion production coinciding with a large drop in microbial abundance, an increase in eDNA and RNA virus abundance. The third week after wet-up, we see the microbial and DNA virus communities 'stabilizing', eDNA abundance slightly decreasing, but a surge in RNA virus abundance. Notably, the virus dynamics described occurred in both the phosphorus-amended and unamended samples, but there were significant differences in the virus communities in the DNA viruses from viromes and the RNA viruses. We further investigated the eDNA by mapping eDNA reads to our other data pools and interestingly, the vast majority of eDNA reads appeared to be microbial in origin. Given the small amount of eDNA that mapped to viruses and the increase in virions detected using viromics post-wet-up, we posit that viral DNA may not persist in soil, indicating that either viral DNA is quickly degraded, or temperate viruses switch to the lysogenic cycle when soil moisture is low and then are induced post wet up. These results also suggest that eDNA accumulates over the dry summer from deceased microbes and after the first rainfall, the eDNA fuels the soil community. Combining multiple meta-omics approaches, CO 2 measurements, and SIP allowed an unprecedented systems-level ecological characterization in these soils improving our understanding of how microbes and their viruses respond to different environmental conditions, and how virus ecogenomics and microbial processes affect the fate of organic carbon.

Bacteriophages | Phage Biobanking | Standard Operating Procedures | Data Application | Cloud Storage | Microbiology Advancements

Proof & Provenance: A federated biobank data platform for coordinating and sharing phages and results

Jan Zheng [1, 3, 4*], Ben Temperton [2], Jon Iredell [3, 4]

1. Phage Directory
2. University of Exeter
3. Phage Australia
4. Westmead Institute for Medical Research

The purity and origin of bacteriophages are essential factors in microbiology research. While current databases are helpful, they don't fully meet the need for a thorough, easy-to-use platform for phage data. This abstract proposes a new approach to phage biobanking: a published collection of phages. In this system, shared Standard Operating Procedures (SOPs), wet lab data, and bioinformatics data would allow for independent assessment of a phage's quality, moving us closer to the goal of creating "Material Safety Data Sheets" for phages.

Phages are constantly evolving, which makes it necessary to track changes and variations in their characteristics. This includes important details such as transfer agreements, safety notes, host characteristics, and specific notes associated with each phage. These elements add to a phage's origin story, providing a clear record of its unique history and uses.

The proposed phage biobanking system goes beyond the traditional database model. It's a full-stack data application that securely manages and uploads a variety of data and file objects. It offers keyword search capabilities and gives users detailed control over how they can publish and share data. This system combines the easy-to-use nature of note-taking apps with the data-handling capabilities of spreadsheet software and the storage abilities of cloud storage services. It's also designed to be open-source and cost-effective.

The main goal of this project is to create a shareable, collaborative platform for phage biobanking that can be audited. By building each layer of the biobank data application stack, this system aims to improve the efficiency and thoroughness of phage biobanking, paving the way for advancements in microbiology.

phage | microbial genomics | phage-host relationships

Building the World's Largest Phage-Host Interaction Atlas using Proximity Ligation Technology

Jonas Grove [1], Sam Bryson [1*], Yunha Hwang [2], Benjamin Auch [1], Demi Glidden [1], Emily Reister [1], Peter Girguis [2], Ivan Liachko [1]

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Viruses, including bacteriophage and archaeal viruses, are the most abundant form of life on earth (1031). They interact with all life and shape the global ecosystem through their impacts on community composition and horizontal gene transfer. However, phage-host relationships have proven challenging to identify without use of culture-based experiments to generate unambiguous evidence for a phage's presence in a given host. These experiments inherently require that all hosts are culturable, typically restricting the scope and microbial diversity that can be surveyed and limiting our understanding of potentially valuable phage-host relationships.

Proximity ligation sequencing is a powerful genomic method for associating viruses with their hosts directly in native microbial communities. Proximity ligation captures, in vivo, physical interactions between the host microbial genome and the genetic material of both lytic and lysogenic phage. Similar to culturing experiments, these linkages offer direct evidence that phage sequences were present within an intact host cell, thereby establishing a phage-host pair. However, unlike culturing experiments, proximity-ligation methods do not require the propagation of living bacterial cells and unlike single cell sequencing experiments, only capture phage-host interactions inside cells. The combination of intra-phage and phage-host signal enables us to simultaneously deconvolve viral genome bins (vMAGs) directly from metagenomes and to assign microbial hosts to large numbers of vMAGs without culturing.

Our application of this technology to hundreds of complex microbiome samples has yielded thousands of novel phage and archaeal virus genomes with host assignments, as well as large numbers of new microbial genomes. Through broad-scale application of proximity ligation sequencing, we are creating a global-scale database of highly diverse phage-host interactions from samples from across the world. We will present published and unpublished work highlighting the power of this approach in the field of metagenomic discovery.

Session 3: Phage defense

Phage defense | defense systems | antiphage | abortive infection

Characterizing the mechanism of phage defense by the Hna system of *Sinorhizobium meliloti*

Leah Sather [1*], Maryam Zamani [1], Jason Kearsley [1], Kathryn Jones [2], Turlough Finan [1]

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Bacteria employ a variety of systems to protect themselves against the phages that infect them. We previously identified a new phage defense system, called Hna, in the nitrogen-fixing alphaproteobacterium *Sinorhizobium meliloti*. Hna protects against phages from both the *Myoviridae* and *Podoviridae* families, and an Hna homologue from *Escherichia coli* confers protection against several well-studied *E. coli* phages, including T7, T4, and the lambdoid phage HK97. The Hna system consists of a single predicted superfamily 2 helicase/nuclease protein that acts via an abortive infection mechanism, meaning that its prevention of phage replication leads to cell death. In an effort to identify how Hna senses phage infection, we isolated “escape” mutant phages that can replicate in cells with Hna. These escape phages carry mutations in a single-stranded DNA binding protein (SSB). We show that expression of this SSB is sufficient to provoke a host abortive infection response in cells carrying Hna, independent of phage infection. We are now characterizing the molecular mechanism by which Hna causes abortive infection and are searching for other “activators” of Hna from other phages. Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assays indicate that host DNA damage occurs when Hna is activated by phage SSB, suggesting that Hna may cause cell death by cleaving the host genome.

anti-phage defence systems | bacterial genomes | Escherichia coli | co-occurrence | synergy

Co-occurrence and synergism of anti-phage defence systems

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Anti-phage defence systems in bacterial genomes show great diversity within and between species. We examined whether combinations of defence systems are promoted or antagonised based on the effects of anti-phage protection. By analysing the co-occurrence of defence systems in thousands of *Escherichia coli* genomes, we found that positive and negative co-occurring combinations are prevalent. Surprisingly, our experimental findings demonstrate that both types of combinations can provide synergistic anti-phage activities, depending on the specific phage being targeted. Furthermore, we observed no consistent patterns of defence system co-occurrence across different bacterial taxa, suggesting that the selection of strains carrying specific combinations of defence systems is driven by environmental pressures rather than mechanistic incompatibilities between defence systems. Additionally, we characterised the synergy between two defence systems that positively cooccurring at the molecular level, revealing the exploitation of similar functional domains among these defence systems. Overall, our study underscores the intricate interplay among antiphage defence systems within a cell.

CRISPR-Cas attack | anti-CRISPR proteins | archaeal viruses | AcrIIIB2 inhibitor | viral immunity interference | type III CRISPR-Cas immunity

How archaeal viruses inhibit host CRISPR-Cas immunity

Xu Peng [1*]

1. Department of Biology University of Copenhagen

In response to host CRISPR-Cas attack, bacteriophages and archaeal viruses evolved a range of diverse anti-CRISPR (Acr) proteins to inhibit host immunity. Our recent research has focused on inhibitory mechanisms employed by archaeal viruses. I will present our recent data on a type III CRISPR-Cas inhibitor, AcrIIIB2. This viral protein binds to CRISPR-Cas type III effector complex and inhibit the turnover of the complex so that continuous activation of enzymatic activities (e.g. ssDNase, cOA synthesis) can not be achieved. The data not only shed light on how a virus inhibit type III CRISPR immunity, but also point out the importance of complex turnover for type III CRISPR-Cas immunity.

Bacteria anti-phage system | Kiwa operons | phylogenetic analysis | transmembrane regions | phage DNA degradation | RecBCD inhibitors

Kiwa modulates host nuclease activity for anti-viral defense

Thomas C. Todeschini [1*], Yi Wu [2], Ameena Naji [3], Rupavidhya Mondi [4], Franklin L. Nobrega [5]

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Bacteria encode diverse genetic systems that enable them to fight against phage predation. While several of these systems have been thoroughly characterised (such as CRISPR-Cas and Restriction Modification), the molecular mechanism of many remains unknown. Here we study the uncharacterised anti-phage system Kiwa, composed of two proteins, KwaA containing transmembrane domains, and KwaB encoding a domain of unknown function. We studied Kiwa by collecting all Kiwa operons present in the NCBI database and performing a phylogenetic analysis that resulted in distinct clades for both KwaA and KwaB genes. Interestingly, KwaB homologs all contained a DUF4868 domain, whilst KwaA homologs showed diversity in size and number of encoded transmembrane regions (TM-region). We therefore selected Kiwa operons present in different clades to test for anti-phage activity. We found that Kiwa systems can give up to 105-fold protection against specific phage families, with anti-phage activity being lost upon deletion of KwaA or KwaB, and truncation of KwaA. Interestingly, the presence of four TM-regions in KwaA is essential for protection, as reconstitution of four regions in Kiwa operons of two TM-regions resulted in anti-phage activity not previously observed. Phage escape mutants revealed that Kiwa is activated when KwA detects the inhibition of the host RNA polymerase by a phage-encoded protein. This activation triggers the activation of KwaB, which forms oligomers and, through an unidentified process, initiates the degradation of phage DNA via RecBCD. Remarkable, this degradation occurs even when the phage produces RecBCD inhibitors, effectively preventing the spread of progeny within the cell population.

CRISPR-Cas defenses | anti-CRISPR strategies | Acr regulation | DNA binding proteins | anti-CRISPR activity control | new anti-CRISPR group discovery

Anti-CRISPR strategies and their regulation

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To counteract CRISPR–Cas defences, phages have evolved anti-CRISPR (Acr) strategies that can inhibit prokaryotic adaptive immunity. Acrs are frequently encoded in an operon with a conserved anti-CRISPR-associated (*aca*) gene. Aca proteins are DNA binding proteins and regulate their corresponding *acr–aca* loci. We have demonstrated that representatives of most classes of Aca proteins function as repressors. The purpose and molecular basis of this Acr regulation is poorly understood. I will present our work on anti-CRISPR regulation, including unexpected findings into how this regulation contributes to the control of anti-CRISPR activity. Finally, I will describe our discovery and characterisation of a new mechanistic group of anti-CRISPRs encoded by phages and other mobile genetic elements.

Session 4: Phage therapy I

Expanded access | Phage therapy | ESKAPE | Antibiotic resistance

A Retrospective, Observational Study of 18 Cases of Expanded Access Phage Therapy

Austen Terwilliger [1,2], Sabrina Green [3], Justin Clark [1,2], Haroldo Santos [2], Kyle Weesner [1,2], Keiko Salazar [1,2], Saima Aslam [4], J. William Campbell [5], Sarah Doernberg [6], Emily Blodget [7], Michele Morris [8], Gina Suh [9], Karam Obeid [10], Fernanda Silveira [11], Claire Bocchini [12], John Reynolds [13], Julie Katkin [14], Lindsey Cameron [14], Jenny Aronson [15], Andrea Censullo [16], Andrey Filippov [17], Katrine Whiteson [18], Barbara Trautner [19,20], Anthony Maresso [1,2]

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9. Division of Infectious Diseases, Department of Medicine, Mayo Clinic, Rochester, Minnesota
10. University of Minnesota, Department of Medicine, Division of Infectious Diseases and International Medicine, Minneapolis, Minnesota
11. University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania
12. Pediatric Infectious Diseases, Baylor College of Medicine, Houston, Texas
13. Department of Medicine, Duke University School of Medicine
14. Texas Children's Hospital, Houston, Texas
15. Infectious Diseases, Stanford Medicine, Redwood City, California
16. Infectious Diseases, Kaiser Permanente, Panorama City, California
17. Wound Infections Department, Bacterial Diseases Branch, Walter Reed Army Institute of Research, Silver Spring, Maryland
18. Department of Molecular Biology and Biochemistry, University of California, Irvine
19. Michael E. DeBakey Veterans Affairs Medical Center, Houston, Texas
20. Department of Medicine, Baylor College of Medicine, Houston, Texas

The emergence of antibiotic resistance is threatening to undermine modern medicine. Dire predictions claim 10 million deaths per year by 2050 at a cost of trillions to governments and healthcare. The problem is compounded by the inherent adaptability of bacteria in the face of intense selective pressures.

Bacteriophages have been proposed as a possible solution for this crisis. Here, we detail progress following the formation of TAILΦR, a phage center that provides personalized treatment for compassionate-use cases. We report the discovery, characterization, pipeline, and process of phage selection and purification, as well as clinical course and outcomes.

TAILΦR responded to 92 requests for a phage hunt, for which we received 86 clinical isolates and generated 217 novel phages against 12 bacterial species. Most common indications requested were urinary tract infection/prostatitis, left-ventricular assist device infections, and bacteremia. From those 86 cases, 18 patients received treatment. Several patients demonstrated bacterial eradication (8/18) and/or clinical improvement (10/18) up to a year post-treatment.

The true potential of phage therapy will not be realized until discovery, manufacturing, and regulatory efforts harmonize to rapidly deliver personalized cocktails in a manner that curtails real-time evolution. TAILΦR is addressing barriers by developing good manufacturing practices (GMP) to significantly reduce time-to-treatment and curating cocktails that anticipate bacterial resistance. Worldwide networks of phage centers will fill a critical treatment void while fueling antibacterial innovation for the most complex and challenging patient cases. This work was funded by U19 AI157981, Robert and Helen Kleberg Foundation, Mike Hogg Foundation, and BCM seed funds awarded to A.M.

phage therapy FDA clinical trials safety effectiveness regulatory pathways

Regulation of Phage Therapy by the FDA: CMC topics

Scott Stibitz [1*]

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For phage therapy to advance to more systematic applications, it is generally accepted that data demonstrating safety and effectiveness, derived from controlled clinical studies, will be required. Such trials are underway (see clinical.trials.gov) under the auspices of the FDA, in the US. In addition to these controlled trials, phage preparations are being used under the FDA's expanded access provisions (often referred to as compassionate use). FDA has been reviewing such submissions for many years now and has found that, although phage therapy presents unique challenges in some ways, existing regulatory pathways, informed by the available scientific data on phage and phage therapy, have been sufficient to these tasks, and have allowed phage therapy studies to progress. In FDA's review of phage therapy submissions, Chemistry, Manufacturing, and Control (CMC) information is crucial. This talk will present i) the agency's current expectations regarding the CMC information that is necessary to provide assurances that a phage product used in humans will be safe and will stand the best chances of being effective; and ii) how this information is expected to develop as a product moves through the stages of clinical development. Topics such as desirable characteristics of phage used for treatment, host strain selection, methods of purification, identification of, and control of, impurities, and stability assessment will be discussed.

Session 5: Phage therapy II

Phage therapy | Informatics | Phage manufacturing | Quality control

Creating systems to safely scale phage therapy, from Phage Directory to Phage Australia

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Phage Directory was founded to help patients access phages by crowdsourcing them from labs worldwide. Over the past six years, we've helped cultivate a community of labs and researchers who share phages and expertise, leading to hundreds of phages shared on behalf of 50+ patients and their physicians, and resulting in at least four successful patient treatments. The first of these was in Australia, which led us to spend the last two years working with the Phage Australia team to help implement a new phage therapy system from the ground up. Their newly accepted STAMP protocol provided a unique framework to collect phage and bacterial data and combine it with clinical outcome data, where each patient would receive their own set of phages.

In 2022 we moved to Australia and began helping build a system for preparing and tracking safe phage preparations in a way that satisfies local and national regulators. We have realized that while the phage community has spent decades collectively published methods on handling the physical aspects of phage therapy, such as phage isolation, characterization, sequencing, diagnostics, production, purification, and quality control, and has collectively amassed thousands of phages against dozens of pathogens, one challenge that has remained overlooked has been managing the information flow, timing, and communication requirements of the phage therapy process. For even one patient, it can require 150+ hours of work to manage phage screening results and therapeutic batches, track patient treatments and outcomes, and coordinate between labs, clinics, and ethics committees while keeping all stakeholders informed at each step and ensuring accurate capture of information throughout the process.

To address these challenges, we've developed phage information management and exchange tools, such as Phage Atlas, and are now exploring the use of large language models like ChatGPT to rapidly extract insights from unstructured lab data to speed up the process even more. Looking ahead, we aim to build tools that can help any phage lab scale their phage therapy process to more patients safely, without overwhelm and without substantial investment. Our goal is to make phage therapy more accessible and scalable, so that effective infection control can become a reality for more patients in need.

Clinical Trial | Systematic Review | Phage Therapy

Phages in Canada

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Canada has a unique historical and now modern contribution to global phage therapy. Over 100 years ago Felix d'Herelle started his scientific career on attempting to distill whisky from maple syrup before exploring and naming the bacteriophage phenomenon. Contributions by authors including d'Herelle in the Canadian Medical Association Journal populate the 1930's. d'Herelle's continued to help launch phage therapy centres internationally. In the 1980's a bacteriophage reference centre at the University of Laval was named in honour of d'Herelle and curated by Professor Ackermann initially. This centre provides phages to over 80 countries.

The modern Canadian roots for phage therapy include Dr Stephanie Strathdee a University of Toronto trained epidemiologist who would launched the modern era through advocating and coordinating the care of her husband. Many other Canadians have gone on to support Phage biology globally.

Nationally, phage therapy launched with Dr Strathdee's talk at the Canadian National infectious diseases and microbiology conference in 2021. This directly led to creation of the Association of Medical Microbiology and Infectious Disease (AMMI) Canada pan-Canadian steering committee and phage therapy work group with Dr Greg German as the chair.

During the COVID-19 pandemic, Phage Canada was created as a scientific meeting which would become a non-profit in 2023.

In 2022, the first modern case of phage therapy was done through the University of Calgary in collaboration with the Hatfull Lab at the University of Pittsburg

2023 is looking to be the year of the phage in Canada as far as phage therapy is concerned. A 5 million dollar (CAD) anonymous gift was provided to the University of Toronto with funds provided to the University of Laval reference centre to expand. In addition, the first registered clinical trial provided phage therapy to successfully treat a chronic urinary tract infection in Toronto with a novel minimally invasive approach.

Continuing the work on urinary tract infection is a registered systematic review of phage therapy with a global one health focus including all years, all languages, and all animals.

The industry in Canada for phage therapy is still in its infancy with some Good Manufacturing Practices production and collaborations internationally.

Finally, Global Clinical Phage Rounds was started in late 2022 supported by the Mayo Clinic, Phage Australia, European Society of Clinical Microbiology & Infectious Diseases, and AMMI Canada. Dr. German is the chair of the network of 100 plus phage clinicians who discuss complex phage therapy cases across the world.

There are inherent regulatory and logistic hurdles for Phage Therapy in Canada but with international support from Houston to San Diego to Sydney to Bangladesh and points in between we have momentum for the future. Canada and Canadians abroad have a unique opportunity to help lead the treatment of antimicrobial resistance pandemic with Phage Therapy as a counter measure.

Session 6: Understanding & improving phage therapy

Phage therapy | phage resistance | antibiotics | *Pseudomonas aeruginosa*

Making Antibiotics Great Again: Phage resistance *in vivo* correlates to resensitivity to antibiotics in pan-resistant *Pseudomonas aeruginosa*

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Phage therapy has a long-standing history in Eastern Europe. In the West, since 2007 the group at the Queen Astrid Military Hospital (QAMH) in Belgium has been treating patients with phage therapy utilizing protocols based on the experiences of the George Eliava Institute of Bacteriophages. A retrospective analysis was performed on a 100 of these cases which included an assessment of phage-resistant bacteria isolated from some of these patient cases. We received 21 isolates from 5 patient cases with varied infections, but all caused by the pathogen *Pseudomonas aeruginosa*. Whole genome sequencing was performed on these isolates combining Illumina (long-read) and Nanopore (short read) technology in order to determine single-nucleotide polymorphisms (SNP). Analysis of sequencing results show changes in phage-resistant isolates recovered from patients correlates with changes in known phage receptors. For one case in particular we see changes associated with resensitivity of pan-resistant bacteria to antibiotics which was demonstrated in patient antibiogram results. This works adds to further evidence that combined phage and antibiotic therapy can be a successful strategy to treat patients with resistant or difficult-to-treat infections.

Pseudomonas aeruginosa | phage therapy | wound infection | murine model | immune system

Topical phage treatment of a *P. aeruginosa* mouse wound-infection model

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Pseudomonas aeruginosa is an opportunistic pathogen that is correlated with persistent infections within wound environments. These infections are often recalcitrant to both antimicrobial treatments and host immune defenses. Development of novel therapies therefore is critical. Herein, we demonstrate the therapeutic potential of well characterized phages, PEV2 and φKMV, as promising strategies for controlling *P. aeruginosa* infections in a murine wound infection model. We also focus on the interactions between phages, bacteria, and immune system that currently are unclear but important for the understanding and development of phage-based therapies. Phage φKMV monotherapy improved *P. aeruginosa*-infection outcomes by decreasing bacterial burden within the wound bed 72 h post-infection by 4 logs and 3.4 logs for the PAO1 reference strain and a clinical wound isolate, respectively. Phage PEV2 reduced PAO1 infection by 2.9 logs but reduced the wound isolate burden by only 1 log. When phage-phage combination therapy was applied, the two phages seem to have interfered with each other as the bacterial burden of wound isolate was reduced by only 2 logs. Immunologically, untreated wound neutrophil numbers were found to be substantially elevated in both the blood and wounds of infected mice, while phage treatment lowered those levels. This suggests that phage-related decreases in bacterial burdens result in lower neutrophil recruitment. Improved healing and overall reduced levels of inflammatory markers were also observed with phage treatment in comparison to untreated wound infections. This was inferred from the correlation of treatment with eradication of bacteria and downregulation of the activity of cells engaged in proinflammatory processes. Histopathology analysis of the PAO1-infected wound tissue showed necrosis extending from remaining superficial dermis to the panniculus carnosus, while phage treatment presented reduced necrosis. We additionally investigated bacterial resistance to phages as they evolved during treatments. Different types of bacterial mutants arose due to changes in transcription regulation, type II secretion, and DNA gyrase function as well as flagellar and LPS biosynthesis, among others. Currently we are studying metabolomic changes within the blood and wound bed during infections and treatments. This proof-of-concept pre-clinical study will provide a platform for translation to human studies targeting *P. aeruginosa* and other problematic multidrug-resistant wound pathogens as well as provide a unique perspective on microbial dynamics and metabolic response to infection and phage therapy. Keywords: phage therapy, Pseudomonas, wound model

Bacteria anti-phage system | Kiwa operons | phylogenetic analysis | transmembrane regions | phage DNA degradation | RecBCD inhibitors

Pf bacteriophage hinder sputum antibiotic diffusion via electrostatic binding

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Chronic *Pseudomonas aeruginosa* (*Pa*) infections remain a leading cause of morbidity and mortality in patients with cystic fibrosis (CF). *Pa* is problematic partly due to its production of robust biofilms, slimy communities of bacteria, and polymers that prevent the diffusion of antibiotics.

We have identified novel roles for filamentous bacteriophages, viruses produced by bacteria, in *Pa* biofilms. We reported that Pf acts as a structural element in *P aeruginosa biofilms* and enhances biofilm function by reducing the antimicrobial effects of tobramycin, thereby contributing to antibiotic tolerance. However, the mechanism of how Pf phages promote antimicrobial tolerance and how this interaction can be targeted for therapeutic purposes is unclear.

Here, by using fluorescent recovery after photobleaching (FRAP), we demonstrate that Pf4 interacts with anti-Pseudomonal antibiotics via charge-based electrostatic interactions. This interactive behavior is enhanced when Pf phages form highly organized structures with CF sputum polymers.

Finally, to target the interaction between Pf and antibiotics, we identified a commonly found antimicrobial peptide in the human airway that can enhance antibiotics penetration through Pf-positive biofilms and increase the bacterial killing efficacy of tobramycin treatment.

Antimicrobials bacteriophage phage therapy urinary tract infection genetic engineering bacteriocin endolysin

Enhancing bacteriophage therapeutics through *in situ* production and release of heterologous antimicrobial effectors

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Bacteriophages operate via pathogen-specific mechanisms of action distinct from conventional, broad-spectrum antibiotics and are emerging as promising alternative antimicrobials. However, phage-mediated killing is often limited by bacterial resistance development. Here, we engineer phages for target-specific effector gene delivery and host-dependent production of colicin-like bacteriocins and cell wall hydrolases. Using urinary tract infection (UTI) as a model, we show how heterologous effector phage therapeutics (HEPTs) suppress resistance and improve uropathogen killing by dual phage- and effector-mediated targeting. Moreover, we designed HEPTs to control polymicrobial uropathogen communities through production of effectors with cross-genus activity. Using phage-based companion diagnostics, we identified potential HEPT responder patients and treated their urine *ex vivo*. Compared to wildtype phage, a colicin E7-producing HEPT demonstrated superior control of patient *E. coli* bacteriuria. Arming phages with heterologous effectors paves the way for successful UTI treatment and represents a versatile tool to enhance and adapt phage-based precision antimicrobials.

phage-specific antibodies | serological profiling | PhageScan | IgG

PhageScan: identification of major phage epitopes that are immunogenic for humans

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Antibodies specific to phage virions have been observed many times, both in animals and in humans. Phages induce the T-dependent type of immune response, which is fundamental for immunological memory and long retention of abilities to recognize and respond to foreign epitopes, as manifested by efficient induction of phage-specific IgG.

In this work we seek to understand what are molecular determinants of phage immunogenicity. Specifically, what are immunogenic phage epitopes, located in what types of proteins, where in phage virions, and possibly linked to what characteristic phage groups?

We conducted serological profiling in humans for specific response to phageome: PhageScan. PhageScan employed a library displaying proteome-wide coverage of peptides from all types of bacteriophages (almost $3x10^5$ oligopeptides) followed by immunoprecipitation and massively parallel DNA sequencing of a library, in EU and US populations. We identified approx. $2x10^4$ oligopeptides that were recognized by human IgG (at differentiated levels), with relevant phage, protein, and source identifications.

PhageScan is the first high-throughput approach that reveals major immunogenic phage epitopes affecting human populations in a natural way, that is by a natural contact with human phageome and/or with phages circulation in the environment. This observation highlights phage groups, proteins, oligopeptides that are “highly visible” for human immune system, thus supporting efficient selection of phages for therapeutic use, and improving our understanding of natural phageomes.

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antibiotic resistance | phage therapy | bacteriophages | targeted phage isolation | cell-to-cell heterogeneity | therapeutic potential of phages

Targeted single phage isolation reveals significant cell-to-cell variations in infection dynamics of phages

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Without action, antibiotic resistance could result in the death of 10 million people by 2050. Fortunately, phage therapy offers an alternative solution that uses bacteriophages or phages to fight bacteria. However, only phages with specific characteristics are suitable for therapy. They must be efficient, toxin-free, and lacking virulence factors. Isolating these specific phages can be challenging and labor-intensive. Yet, we have developed a targeted phage isolation method that enables the screening of suitable phages from both environmental and human-derived samples. In addition, we can evaluate their efficacy on both the population and single-cell levels. By using this method, we have also discovered significant cell-to-cell heterogenicity that varies depending on the type of phage tested. Going forward, it would be interesting to evaluate the effect of this variation on the therapeutic potential of phages.

Nanotweezers | single-cell | phage | susceptibility | testing

Optical Trapping of Bacteria for Ultrafast Bacteriophage Lysis Detection at the Single-Cell Level

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Regarding their high bacterial strain specificity, rapid and accurate selection of therapeutic bacteriophages is crucial in phage therapy clinical protocols. Here, we report the use of photonic crystal cavities as on-chip nanotweezers for ultrafast phage susceptibility testing (PST) at the single bacterium level. We used an integrated optical trap based on silicon hollow photonic crystal (HPhC) nanocavities to trap a single *Escherichia coli* B cell. The optical cavity has a characteristic resonance wavelength, which is modified when an object is trapped. This translates in a transmission jump DT. The collected transmitted signal depends on the characteristics of the trapped entity. *E. coli* cells were put in contact with T4 *Myoviridae* phages before being injected in the trapping device. We report direct observation of a bacterium-phage lytic event in the optical cavity. Bacterial burst likely allows the liquid medium to enter the cytoplasm, which implies reduction of the refractive index contrast between the bacterium and the medium. The resonant mode of the cavity being less perturbed, the transmission signal decreases. Accordingly, only 40min ± 5min after the mixing of phages and bacteria (t = 0), we observe an abrupt decrease of the transmission signal correlating with the bursts of the trapped bacterium. The detection of the lysis event is much faster than current culture-based phagograms requiring 16h-24h incubation times. This innovative phagogram approach paves the way to ultrafast PST at the single bacterium level.

in vitro model | PK/PD | phage cocktail | bacterial resistance | population dynamics | Staphylococcus aureus

A Preclinical Two-Compartment Model of Localized Infections to Study the Effects of Phage Dosing Parameters on Bacterial Killing and Resistance Development

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Infections that do not respond to antibiotic therapy present challenges to public health. Phages are being widely studied as a potential non-antibiotic treatment for such infections. However, because phages replicate in the presence of their target, typical PK/PD analyses have limited ability to inform our use of them, particularly dosing.

The Hollow Fiber Model (HFM) is a 2-compartment in vitro model that we use to represent a localized infection and a circulatory system so that we can investigate which system parameters have the largest effect on experimental outcomes such as bacterial killing and phage resistance. The bacteria are confined to a 3.9 mL space filled with porous fibers that allow a continuous exchange of nutrients and phages between compartments. *S. aureus* populations are allowed to reach a steady-state concentration of approximately 1×10^{10} CFU/mL, then we add phages to mimic 1×10^{9} PFU being diluted into a 5 L adult blood volume. The circulating compartment and bacterial compartment are monitored for 3 weeks to quantify phage and bacterial populations.

Experiments were conducted at 37°C using a methicillin-sensitive strain (ATCC 19685) and a USA300 MRSA strain (NRS384). Our initial experiments tested the bactericidal effects of three different phage-dosing regimens when using a single phage: either phage K or a *gp102* mutant that exhibited improved antibacterial activity on USA300 MRSA strains in previous studies. Each of these phages caused a transient 2-3 $\log_{10}$ reduction in CFU/mL of ATCC 19685, which rebounded after 3 days; neither phage affected NRS384 populations. Phage concentrations were consistently higher in the bacterial versus the circulating compartment. We then used host-range and cross resistance data to design three phage cocktails, which we tested using the most intensive phage dosing regimen from the single-phage experiments. The cocktail experiments resulted in the same bactericidal outcomes as the single phage experiments. However, with the USA300 MRSA strain in particular, we saw a lot of variability in which individual phages became most abundant or persisted in the bacterial compartment, suggesting phage competition. Preliminary characterization of bacterial populations at different timepoints showed a mixture of phage-sensitive and phage-resistant cells. We are continuing to explore the effects of variables such as bacterial strain choice, phage treatment, dosing regimen, and media composition and flow rate and are working to relate these findings to observations from animal data and data from other *in vitro* models.

bacteriophage | cocktail | pharmacokinetics | phage-host interaction | phage therapy | time-kill kinetics | dose | cocktail design

Lost in circulation: Improving the *in vivo* activity of phage cocktails

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Phage research has considerably intensified in the last decade, yet clinical phage therapy still proceeds with mixed success. Therapeutic phages are optimally given as cocktails of two or more phage strains, each targeting different host binding receptors. While refining cocktail design for phase I/II clinical trials, strong biological rationale is required for the inclusion of each phage in formulation. Currently, we cannot ensure that all phages given during phage therapy are active *in vivo*. Moreover, a major hurdle in cocktail design lies in the fact that phages are selfish elements that interfere with infection by other strains. This suggests that phages in combination are unlikely to exhibit synergism and can only interact additively or antagonistically. We determined the lytic activities of *Pseudomonas aeruginosa* 2-phage cocktails *in vitro, in silico*, and in patient. We measured the activities of three phages administered as single, 2-phage simultaneous, or 2-phage sequential treatments in well-mixed *in vitro* time-kill microcultures. We observed the greatest lytic activity (lowest OD_{600}) from 2-phage simultaneous treatments. Applying mathematical models to the time-kill kinetics, we predicted that the two phages additively improved bacterial lysis by up to 15%. The increased lytic activity also promoted more robust growth of both phage strains over a longer period of time. Next, we mimicked the spatial complexity and hydraulic pressures of an *in vivo* circulatory system using an *in vitro* model. In the hollow fiber infection model (HFIM), 2-phage cocktails additively reduced bacterial numbers for a longer period of time (>24h) compared to single phage (<12h). In contrast, phage numbers suggest antagonism between phage strains. Replication of one of the two phage strains was delayed for up to 12h in the HFIM. Returning to time-kill microcultures, the early timepoints (0-3h) of 2-phage simultaneous treatment showed reduced lytic activity from cocktail treatment compared to singular phage activity. Combined, this suggests that the lytic activity of phage cocktails are additive at the population level but antagonistic at the cellular level. *In vivo*, however, host factors can interfere with the additive properties of cocktails. In patient, this was observed during intravenous 2-phage cocktail treatment of *P. aeruginosa* pneumonia. Lung sputum metagenomes collected on days 4, 8, and 13 of treatment showed high relative abundance of only one of the two treatment phages. Despite both phages being administered twice daily at equal ratio, this suggests that only one phage strain was lytically active *in vivo*. Together this suggests that when designing phage cocktails, it is important to dose phages based on each strain's lytic activity to provide the greatest additive effect. A strain-centric view of phage cocktails may therefore improve the *in vivo* success of therapy.

annotation genome bioinformatics

Enhancing phage therapy safety: Reliable and sensitive phage genome annotation with rTOOLS2 high-throughput pipeline

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Phage therapy is an exciting and promising approach to fight bacterial infections. However, ensuring the safety and efficiency of phages for therapeutic use requires a thorough understanding of their genomic properties. This process enables detection of genes that make phages potentially harmful for the subject of the therapy or the environment, such as antibiotic resistance, lysogeny, and virulence genes.

Traditional bioinformatics tools designed for bacterial genomes are not well-suited for phage genomes due to their unique structure, leading to poor gene calling and function annotation. Recently, phage-focused tools have been released, such as Pharokka and rTOOLS2. rTOOLS2 is a multi-hypothesis, phage-focused annotation pipeline: its advanced algorithm uses the output produced by widely-used annotation tools to find more gene functions, with high evidence thresholds to avoid false positives.

In this study, 135 phage genomes published in Genbank were annotated using Pharokka and rTOOLS2's high-thoughput version, and the results were compared.

Pharokka was able to improve the average published annotation, as the average number of genes functionally annotated grew from 29.5% to 35.9%. On the other hand, rTOOLS2's high-throughput version was able to significantly increase the rate of annotated genes, reaching 54.6%.

To promote the safest possible use of phages for patients and for the environment, it is key to use thoroughly characterized phages. rTOOLS2's high-throughput version can rapidly provide a strong basis for genome characterization. The use of curated databases ensure that meaningful annotations are provided, and results can be published with low risk of public database poisoning. Moreover, rTOOLS2 is able to produce more information, as it nearly doubled the number of annotated genes in the initially published genomes.

Enterococcus infections | phage therapy | antibiotic resistance | exopolysaccharide synthesis genes | phage-resistant mutants

Better together: Phage cocktails constrain the growth of Enterococcus

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Due to the rise in antibiotic resistance, *Enterococcus* infections are a major health crisis that requires the development of alternative therapies. Phage therapy offers an alternative to antibiotics and has shown promise in *in vitro* and in early clinical studies. Here we isolated 18 *Enterococcus* phages from Southern California wastewater, characterized their host range against a collection of 19 clinical *E. faecalis* and *E. faecium* isolates, and sequenced their genomes. We showed that cocktails of two or three phages often prevented the growth of phage-resistant mutants, and identified which phages were replicating the most in each cocktail. When resistant mutants emerged in response to single phages, they showed consistent accumulation of mutations in exopolysaccharide synthesis genes. These data serve to demonstrate that a cocktail approach can inform efforts to improve efficacy against *Enterococcus* isolates and reduce the emergence of resistance.

Phage therapy | Directed evolution | Drug-resistant Pseudomonas aeruginosa

Improvement of a therapeutic phage cocktail against multidrug-resistant *Pseudomonas aeruginosa* using directed evolution

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Antibiotic resistance represents a growing and serious global challenge, with clinicians struggling to provide effective antibacterial treatments. Multidrug-resistant (MDR) strains of *P. aeruginosa* are spreading throughout the world and cause especially difficult-to-treat infections, with antibiotics losing efficacy. Phage therapy represents a powerful tool to supplement or possibly replace failing antibiotics for the treatment of these MDR infections. Phages offer attractive characteristics for therapeutics, including replication at the site of infection and minimal off-target killing because of the high strain specificity of most phages. However, such tight specificity makes development of durable, off-the-shelf phage cocktail therapeutics with broad killing spectra difficult to generate. In this work, we employed an *in vitro* directed evolution approach (the Appelmans protocol) to develop phages with broader host ranges against a panel of global, diverse MDR clinical strains. **As a result we generated, isolated and characterized a phage clone with expanded host range and incorporated it into a phage cocktail that provided therapeutic efficacy in a mouse model of wound infection.**

We employed the phage training approach known as the Appelmans protocol, in which we mixed three genetically similar phages with complimentary lytic activity and passaged against a 12-strain panel of *P. aeruginosa* phage-resistant clinical isolates and a phage-permissive host. Following multiple rounds of phage training, we were able to isolate phage clones with altered and/or expanded host ranges when tested against a 100-strain global diversity panel of MDR *P. aeruginosa* clinical isolates. Altogether, we isolated 10 phage variants that were plated on a previously pan-phage-resistant clinical strain of *P. aeruginosa*, MRSN 20176. Of these 10 phages, six had expanded host range, while the remaining four showed host ranges either roughly equal to or reduced compared with the parental phages. We sequenced the phage clones and characterized recombination and SNP events. Upon deeper analysis, we observed four major sites of recombination and the presence of numerous SNPs when compared with the most closely similar parental phage genome. We identified point mutations in genes predicted to encode tail fibers. We additionally identified mutations and recombination events in genes predicted to encode DNA polymerase and RNA polymerase and other phage structural proteins. We tested the stability of phages with expanded host ranges to determine whether the observed spectral expansion would be retained after repeated propagation on a single host. Phage clones 20176-4, 20176-5, 20176-6, and 20176-7 were passaged against phage-permissive strain PAO1 serially over five rounds. Lysates were then plated, and three clones were collected from each, purified, and re-plated against the 100-strain global diversity set to assess host range. Of these, phage subclones collected from 20176-4 showed an increase in host range, while the remaining clones showed either retention of host range or a reduction. We selected phage subclone 20176 4-2, a subclone of 20176-4, for use in phage cocktail development. Phage 20176 4-2 was assessed for compatibility and stability in mixes with other top performing phages from our library, assessed for *in vitro* killing efficacy, and then, endotoxin was removed from the preparation for incorporation into a phage cocktail. The phage had stable titer over two months of storage, was compatible with other cocktail phages, and rapidly killed susceptible *P. aeruginosa* strains. A new phage cocktail containing the trained phage was designated PAM3T. PAM3T was derived from therapeutic phage cocktail WRAIR_PAM3, which consists of six *P. aeruginosa* phages and has a combined host range of 83% of the global diversity panel. We removed two components of WRAIR_PAM3 and replaced them with 20176 4-2. PAM3T consists of only five phages but has expanded activity, with a combined host range of 85% of the diversity panel. PAM3T was then tested in a mouse dorsal wound model of infection with *P. aeruginosa* strain PAO1::*lux*, with four phage doses provided daily both topically and intraperitoneally. While 7/8 (87.5%) of control mice treated with saline died, the phage treatment protected 100% of mice from lethal septicemic infection. PAM3T reduced the burden of *P. aeruginosa* PAO1::*lux* in infected wounds to undetectable levels within two days, and phage-treated wounds closed as rapidly as by Day 14, versus by Day 21 for positive control mice treated with ceftazidime.

In conclusion, we demonstrated that a directed *in vitro* evolution approach can yield phages with expanded or altered host ranges, and that these expanded host range clones can provide powerful additions to therapeutic phage cocktail design by reducing the number of phages required for a broad host range cocktail and by providing activity directed at phage-resistant strains or clonal groups. Our trained phage 20176 4-2 had a stably expanded host range and compatibility with top performing phages in our library, and phage cocktail PAM3T that included this phage showed high therapeutic efficacy in a mouse wound infection model.

phage diagnostic bacterial infection

Circulating bacteriophages can identify bacterial pathogens in human infections

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Bacteriophage, viruses that infect bacteria, have great specificity for their bacterial hosts at the strain and species level. However, the relationship between the phageome and associated bacterial population dynamics is unclear. Here, we generated a computational pipeline to identify sequences associated with bacteriophage and their bacterial hosts in cell-free DNA (cfDNA) from plasma samples. Analysis of two independent cohorts, including a Stanford Cohort of 62 septic patients and 10 controls and the SeqStudy cohort of 224 septic patients and 167 controls, reveales a circulating phageome in the plasma of all sampled individuals. Moreover, infection is associated with overrepresentation of pathogen-specific phages, allowing for identification of bacterial pathogens. We find that information on phage diversity enables identification of the bacteria that produced these phages, including pathovariant strains of Escherichia coli. Phage sequences can likewise be used to distinguish between closely-related bacterial species such as Staphylococcus aureus, a frequent pathogen, and coagulase-negative Staphylococcus, a frequent contaminants such as. Phage cfDNA may have utility in studying bacterial infections.

Session 7: Phage and the immune system

immune system | macrophages | phage resistance | trade-offs

Antagonism between bacteriophages and macrophages increases bacteriophage resistance and inhibits evolution of bacteriophage infectivity

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Phage therapy, the use of viruses that infect bacteria (bacteriophages), is being trialled as a complement to antibiotics during the antimicrobial resistance crisis. While bacteria can evolve bacteriophage resistance, bacteriophages can evolve to increase their infectivity against resistant bacteria (coevolution). Additionally, bacteriophage resistance is hypothesised to be beneficial in phage therapy by increasing bacterial susceptibility to the immune system. Trade-offs between immune and bacteriophage susceptibility may affect bacteria-bacteriophage coevolution by increasing the costs of acquiring more resistance mutations and reducing mutation rates. Here, we examine how a pathogenic bacterium, *Pseudomonas aeruginosa* coevolves with two clinically relevant bacteriophages (14-1 and PNM) when in the presence of macrophages (RAW 264.7 cell line). Bacteria were shown to have increased survival in the short-term when macrophages were present, leading to increased rates of bacteriophage resistance over one week. Macrophages inhibited bacteriophage infectivity evolution without phagocytosing bacteriophages. We hypothesise that the spatial structuring introduced by macrophages may reduce bacteria-bacteriophage population mixing, slowing rates of coevolution and leading to increased bacterial resistance. These results have implications for our understanding of the immune-system's role in influencing phage therapy outcomes.

bacteriophage macrophage inflammation phage-human interaction anti-viral IFN-beta

The Viral Immunogenicity of Clinically Relevant Bacteriophages

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Multiple curative phage therapies are being developed to counter multidrug resistant diseases. The leading candidates are now being evaluated in human phase 1 and/or phase 2 clinical trials. Several single patient phage therapies have already concluded, while many others will advance in the future. Phages have been increasingly recognized for their influence on the human immune system including bacterial disease, and may also influence the outcomes of phage therapies. We have analyzed the immunogenicity of Pseudomonas aeruginosa phages in vitro, in animal, and in patient. We quantified the upregulation of 410 genes and downregulation of 206 genes in macrophages exposed to myovirus PAK_P1. This correlated with increased production of several proinflammatory cytokines, including IFNβ, TNF, CXCL1/KC and IL-17A. Peak expression was observed after six hours of phage exposure. Applying gene ontology, we found that macrophage response was largely driven by transcription factor NF-κB, promoting expression of predominantly antiviral responses. We determined that extracted phage DNA induced a similar cellular response to that observed with whole phage particles. In contrast, bacterial LPS toxin induced a differential cytokine. This suggests that TLR9 is largely involved in sensing phage DNA. We observed that TLR2 may also be involved in phage capsid sensing by macrophages. Next, we found that phages in the lungs of mice caused exposed mice to lose significant weight. Weight loss was comparable to airway exposure to LPS toxin. Phage PAK_P1 has also been used to treat infectious disease in humans. Post-treatment analysis of patient serum suggests that 14 days of twice daily treatments promoted generation of phage specific neutralizing antibodies. Applying an antiviral-based framework to our findings indicated parameters that may inform the development of more effective, safer phage therapies in clinical practice.

Deimmunization peptidoglycan hydrolases endolysin S. aureus infections preoteintherapeutic

Deimmunization of peptidoglycan hydrolases for therapeutic treatment of systemic *S. aureus* infections

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Staphylococcus aureus is an opportunistic pathogen colonizing roughly 30% of the human population, causing a wide range of diseases. Due to the emergence of resistant strains and the lack of new antibiotics, novel antimicrobials are of high interest. Phage-derived peptidoglycan hydrolases (PGH) could be used as a treatment against drug-resistant bacterial strains. Fast lysis, high specificity and activity against drug-resistant bacteria are just a few advantages for the use of PGHs as protein therapeutics. A major drawback of protein drugs is the immunogenicity of foreign proteins. T cells play a key upstream role in the activation of the adaptive immune system and therefore, the immune reaction against protein therapeutics. Antigen presenting cells sample the environment and proteolytically process proteins, which are then presented as T cell epitopes on MHCII on the cell surface. This leads to the activation of CD4 T cells inducing the activation and differentiation of other T and B cells and with this the production of highly specific and long-lived anti-drug antibodies. Therefore, deimmunization approaches usually focus on T cell epitope prediction and deletion. This project applies computational tools to predict T cell epitopes and design variants with epitope deleting mutations with low impact on protein activity and structure. Deimmunized variants undergo *in vitro* activity testing, *ex vivo* immunogenicity assays and *in vivo* immunogenicity and efficacy studies in humanized mice.

Tuberculosis | mycobacteriophages | humoral immune responses | intravenous delivery | aerosol delivery

Host humoral immune responses to mycobacteriophages after repeated intravenous or aerosol delivery in a preclinical mouse model

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Tuberculosis (TB) kills more individuals globally than any other single infectious disease. Health care professionals and household contacts in endemic areas have routinely high *Mycobacterium tuberculosis* (M.tb) pathogen exposures. Focused intervention strategies designed to block transmission in these populations would help to significantly reduce the burden of TB disease. Mycobacteriophages (phage) are an underutilized biologic intervention for the pathogen M.tb and could be leveraged for their bactericidal properties. Our goal was to address outstanding questions about the utility of phage therapy against M.tb. Specifically, we determined the impact of repeated mucosal or intravenous (i.v.) exposures to anti-M.tb phage Fionnbharth*Δ45Δ47* (Fionnbharth), an engineered lytic phage on host anti-phage immunity in a preclinical mouse model. We hypothesized that repeated i.v. phage delivery would induce high titers of functionally neutralizing anti-phage antibodies, but mucosal delivery via the aerosol route would not. To evaluate the impact of repeat exposures on host anti-phage immunity, male and female C57BL/6 mice were dosed 6 times weekly with Fionnbharth i.v. or via aerosol, and serum and bronchoalveolar lavage fluid (BALf) were collected weekly. The anti-phage antibody magnitude and endpoint titer (EPT) for each condition and timepoint was determined using an in-house developed ELISA. We observed that repeated i.v. phage dosing induced a significantly higher magnitude of anti-phage humoral responses than the same phage delivered via aerosol over the entire time period evaluated. After 6 weekly i.v. doses, anti-phage total IgG EPTs for serum samples reached a magnitude of 5.0 log10 while aerosol cohorts were often below or at the assay's limit of detection (1.0 log10). Endpoint titers of anti-phage total IgG in BALf samples for i.v. dosed mice reached 2.0 log10, while the aerosol cohorts remained below the limit of detection (0.0 log10 EPT). We observed a reverse profile for IgA samples where aerosol treated cohorts had a low but detectable serum EPT (2.1 log10) and i.v. cohorts were at the limit of detection (1.0 log10). Importantly, we demonstrated that serum samples collected after 6 weeks of repeated i.v. dosing contained functionally neutralizing antibodies that were able to inhibit phage-mediated lysis of a representative mycobacteria, *M. smegmatis*. We did not observe the same phenotype from aerosol treated cohort samples. These data suggest that aerosol delivery of phage is a viable approach for therapy because it does not induce a robust neutralizing anti-phage response and should be considered as a prioritized route for further evaluation of efficacy against M.tb.

Phage Therapy | Phage Steering | Immunophage Synergy | Antivirulence Strategies | Burkholderia

O-antigen binding *Burkholderia* phage KS12 controls infections by the emerging pathogen *Burkholderia gladioli* through selection for immunosensitive & avirulent bacterial populations

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The increasing global spread of multidrug resistant (MDR) bacterial pathogens is an imminent menace to public health and threatens virtually all aspects of modern medicine. *Burkholderia gladioli* (Bg), a close relative of the notorious *Burkholderia cepacia* complex (Bcc), has historically been identified primarily as a pathogen of several agriculturally relevant plant species, but has been increasingly recognized in recent years as an opportunistic human pathogen as well. Indeed, Bg is now the third most frequently isolated *Burkholderia*species among American cystic fibrosis patients, and is associated with a particularly poor prognosis. Importantly, Bg - like all *Burkholderia* species - characteristically exhibits a high degree of antibiotic resistance, meaning that novel modes of treatment for infections by this pathogen are urgently required.

One potential alternative is the use of phages, viruses that destroy targeted pathogenic bacteria while leaving commensals and host cells unharmed, in what is called phage therapy. Although Bg is an ideal candidate for phage therapy due to its MDR status and pathogenicity, very few phages targeting this species have been identified. We hypothesized that previously characterized Bcc phages could cross-infect Bg, which could be exploited to develop phage therapy targeting Bg in clinical settings.

To explore this, we quantified the antibacterial effects of a panel of 8 Bcc phages on 12 clinical and environmental strains of Bg *in vitro*, and the most promising candidates were subsequently tested *in vivo* using the *Galleria mellonella* larval model, and *ex planta* using the newly developed *Allium cepa* maceration model. Among the most effective Bcc phages is the lytic myovirus KS12, which has been shown to reduce the *in vitro* growth of Bg strains R406 and R1879 by over 95%, and produces roughly 50% mortality and morbidity reductions in *G. mellonella* and *A. cepa*, respectively. Interestingly, KS12 is unstable in *G. mellonella* haemolymph despite exhibiting a powerful antibacterial effect, and we therefore sought to investigate the dynamics of this fascinating phenomenon.

We identified that KS12 utilizes the O-antigen of bacterial LPS as a primary receptor, and therefore selects for phage-resistant survivor populations with truncated LPS. These survivors have significantly reduced virulence in *G. mellonella* and *A. cepa*, and are sensitized to human complement and several human and insect cationic antimicrobial peptides. Simultaneously, KS12-mediated lysis destroys much of the bacterial population and releases immunostimulatory LPS, thereby recruiting immune cells which can efficaciously target the sensitized survivor population. Together, these mechanisms explain the ability of KS12 to control *B. gladioli* infection despite being unstable *in vivo*, and suggest that phage inactivation may not pose a problem for the therapeutic use of at least some phages. Interestingly, we identified that although KS12 is readily inactivated by human complement, it remains unaffected by murine macrophages - meaning that immune inactivation of phages may only occur in certain tissue compartments. Finally, KS12-resistant survivor mutants are sensitized to polymyxins B and E, to which *Burkholderia* species are almost universally resistant, raising the possibility that these drugs could be used synergistically with KS12, and possibly other phages, to maximize antimicrobial effects.

Our findings demonstrate that Bcc phages can effectively control the growth of Bg *in vitro*, *in vivo*, and *ex planta* and offer fascinating new insights into the complexities of phage-bacterium-host interactions, which collectively suggests that these phages could be used for clinical therapy targeting the emerging pathogen *Burkholderia gladioli*.

Session 8: Molecular phage biology I

LPS | Overcoming host resistance | Genotype-phenotype maps | Experimental evolution | ΦX174 | E. coli C.

Stepwise evolution of *E. coli* C and PhiX174 reveals unexpected lipopolysaccharide (LPS) diversity

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Phage therapy is a promising method for the treatment of multi-drug resistant bacterial infections. However, its long-term efficacy depends on understanding the evolutionary effects of treatment. Current knowledge of such evolutionary effects is lacking, even in well-studied systems. We use the bacterium *Escherichia coli* C and its bacteriophage PhiX174, which infects cells using host lipopolysaccharide (LPS) molecules. We generate 31 bacterial mutants resistant to PhiX174 infection and based on the location of resistance mutations and current knowledge, we predict these produce eight distinct LPS structures. Next, we isolate 16 PhiX174 mutants that can, between them, infect all 31 mutant hosts. Finally, we determine the infectivity profiles of the 16 evolved phages, revealing 14 distinct profiles. Given that only eight profiles are expected if LPS predictions hold, our results demonstrate that the current understanding of LPS biology is insufficient to accurately forecast the evolutionary consequences of infecting bacterial populations with phage.

ROS bacteriophages metagenomic libraries oxidative stress

Functional metagenomic DNA libraries (IMMΦRTL v1.0) screening for the isolation of novel genes against reactive oxygen species

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Reactive oxygen species (ROS) are unstable molecules that constantly threaten the genomic integrity of all domains of life, including bacteriophages. Since ROS can react with nucleic acid, proteins, and lipids, causing permanent damage in a cell, all organisms have evolved protective mechanisms against ROS. These mechanisms range from scavenging toxic radicals to repairing the genome when damaged. Previous findings from our laboratory revealed functional phage-encoded ROS scavengers in uncultured bacteriophage genomes. Thus, environmental bacteriophages are good sources for discovering novel genes involved in protecting against ROS. We subsequently constructed five functional DNA libraries (genomic, multigenomic, and three metagenomic) using various sources of DNA recovered from the environment and cultured bacteriophages. We named these Inducible Multi Metagen*mic RecombinanT Libraries (IMM*RTL) v.1.0, which can be valuable resources for discovering novel biology. Then, we screened IMM*RTL v.1.0 in mutant *E. coli* lacking all major ROS scavengers (*Δcatalases*) for their ability to rescue bacterial mutants from a fatal oxygen radical challenge. Here, we report the overall DNA library construction, the screening process, and the recovery of positive hits that overcame both fatal ROS attacks and genetic defects. These hits may help elucidate novel mechanisms that bacteriophages possess to guard their nucleic acids against ROS and may help shed some light on preventing ROS-associated diseases such as aging and cancer.

Bacteriophage | transmission electron microscopy | scanning electron microscopy | host-phage interaction | biofilm

Isolation and characterization of a novel lytic bacteriophage Sfk20 infecting *Shigella flexneri* and its synergistic effect in combination with ampicillin against biofilm

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Enteric diseases such as Shigellosis are one of the leading causes of childhood morbidity and mortality worldwide. To cure bacterial infections, reckless use of antibiotics has gradually made them resistant to them. Antibiotic-resistant bacterial infections cause an impact on healthcare, food security, and different socio-economic condition in many developing and under-developed countries. The unmet needs of developing and underdeveloped countries are clear as the global antibiotic resistance epidemic has now reached a catastrophic level. The increased prevalence of multidrug-resistant *Shigella* species has revived the importance of bacteriophages as an alternative therapy to antibiotics. Because of the bactericidal properties of lytic bacteriophages, they are considered potential biocontrol and therapeutic agents. As a result, phage research involving isolation, characterization, and applications has recently significantly increased.

In our study, a *Shigella* bacteriophage Sfk20 was isolated from the environmental water of the diarrheal outbreak area of Kolkata and found to be a novel lytic phage with promising potential against the host bacteria *Shigella flexneri* 2a. The morphological study (using transmission electron microscopy and scanning electron microscopy) revealed that the bacteriophage had a prolate head 91.08 ± 4.92 nm (length), 62.34 ± 4.82 nm (width), and a long contractile tail 99.59 ± 4.92 nm (length) 18.66 ± 2.52 nm width and belongs to the myoviridae family. Phage Sfk20 showed infectivity against *Sh. flexneri, Sh. sonnei, Sh. dysenteriae1* and two non-typhoidal *Salmonella* strains. The one-step growth curve study of Sfk20 revealed a latent period (20 mins) and large burst size (123 pfu per infected cell). Phage Sfk20 showed high stability at a 4-37ºC temperature range and at a pH range of 7-9. A study on phage stability conducted at different salinity revealed phage Sfk20 remains active within 0-5% salt concentration. A study on understanding the nature of phage Sfk20 host receptor suggested that the outer membrane LPS of the *Sh. flexneri* 2a acts as a receptor for the phage Sfk20. The bacteriolytic activity of phage Sfk20 at various MOI studies revealed that at high MOI the growth of the host bacteria became restricted. The whole-genome sequencing study revealed that the bacteriophage Sfk20 contains a linear double-stranded genome that consists of 164878 bp, 35.62% GC content 241 ORF, and 10 tRNA. Genomic analysis also confirmed the presence of lytic genes and the absence of lysogeny, virulent, and toxic genes. The comparative genomic study and phylogenetic analysis suggested that Phage Sfk20 belongs to the T4-like virus genus family of Myoviridae and caudovirales order. In proteomic analysis, the LC-MS/MS technique used here for detecting and identifying 40 Sfk20 phage proteins helped us to get an initial understanding of the structural landscape of phage Sfk20. Among the identified proteins, six structurally relevant proteins were selected and their structure was predicted using neural network and template-based modeling software. The structural characterization of the phage Sfk20 was done using single-particle cryo-electron microscopy and image processing. Reconstruction of T-4 like myoviridae phage Sfk20 was performed using EMAN 2.9 and Relion 3.1 software. The structural characterization of phage and its proteins further expands our knowledge of phage biology. The attachment of the phage particle to its host and subsequent intracellular development of phage and host cell lysis were visualized in a time-dependent experiment using thin-section transmission electron microscopy (FEI Tecnai 12 BioTwin) and scanning electron microscopy (FEI Quanta 200). This study further confirmed the lytic cycle of phage Sfk20. Bacteriophage Sfk20 showed antibiofilm activity against *Shigella* bacteria both alone and in combination with ampicillin. The synergistic effect of Sfk20 and ampicillin on the removal of biofilm was visualized by scanning electron microscopy. This study further revealed that the efficacy of the phage could be enhanced by utilizing the antimicrobial synergy between phages and antibiotics. All the results of this study imply that Sfk20 has the potential to be used as a biocontrol agent and phage therapy candidate.

Keywords: Bacteriophage; transmission electron microscopy; scanning electron microscopy; host-phage interaction; biofilm

lysis ssRNA phage high-throughput genetics

Multicopy-suppressor screens reveal convergent evolution of single gene lysis proteins

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In contrast to dsDNA phages where multiple proteins are involved in programmed host lysis, lysis in ssRNA *Fiersviridae* and ssDNA *Microviridae* phages requires only a single gene (*sgl for single gene lysis*) to meet the size constraints of some of the smallest genomes in the biosphere. To achieve lysis, Sgl proteins exploit evolutionary “weak spots” in bacterial cell wall biogenesis. In several cases, this is done by inhibiting specific steps in Lipid II synthesis. Recently metatranscriptomics has revealed thousands of novel ssRNA phage genomes, each of which must carry at least one *sgl* gene. Determining the targets of these Sgl proteins could reveal novel vulnerabilities in bacterial envelope biogenesis and may lead to new antibiotics. Here, we employ a high-throughput genetic screen to uncover genome-wide host suppressors of Sgl activity and apply it to a set of diverse Sgls with unknown molecular targets. In addition to validating known molecular mechanisms, we determined that the Sgl of PP7, an ssRNA phage of *P. aeruginosa,* targets MurJ, the flippase responsible for Lipid II export which was previously shown to be the target of the Sgl of coliphage M. These two Sgls, which are unrelated and predicted to have opposite membrane topology, thus represent a case of convergent evolution. Another set of Sgls which are thought to cause lysis without inhibiting cell wall synthesis elicit a common set of multicopy suppressors, suggesting these Sgls act by the same or similar mechanism.

phage-antibiotic synergy | antibiotic tolerance | dormant bacteria | stress response

Phage Paride exploits bacterial stress responses to kill antibiotic-tolerant cells

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Bacterial infections are a growing health concern worldwide and alternative treatment options are urgently needed to combat this new pandemic. Bacteriophages prey on their hosts irrespective of antibiotic-resistance and have been long recognised as an alternative means of combating infections. However, their unreliable performance *in vivo* despite their high *in vitro* potency has hindered their adoption as a reliable treatment option. Similarly, survival of drug-sensitive bacteria to antibiotic treatment has been linked to persistence of non-growing, dormant cells inside patients and evolution of antibiotic resistance. Given the high abundance of non-growing bacteria in the environment, we reasoned that some phages capable of infecting dormant, antibiotic-tolerant cells should exist. Through extensive screening of environmental samples, we isolated a new *Pseudomonas aeruginosa* phage which we named Paride. Paride can kill over 99% of cells in non-growing cultures harbouring high numbers of antibiotic-tolerant bacteria. Combined with the carbapenem meropenem, Paride led to the sterilization of these cultures, and this synergy was also observed in a murine model of a chronic *P. aeruginosa* implant infection. Furthermore, we show that efficient replication of Paride on dormant hosts depends on the same bacterial stress responses known to drive antibiotic tolerance. Therefore, we propose that Paride exploits vulnerabilities of the dormant physiology of antibiotic-tolerant bacteria and that understanding the underlying molecular mechanisms of these vulnerabilities will inspire novel therapeutic approaches to combat and prevent the emergence of treatment-resistant infections.

Session 10: Ecology & Informatics II

phages ecological insights phage isolation phage characterisation phage therapy antibiotics UTIs

Embracing the complexity of phages using ecological insights to advance phage therapy

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Unlike a standard antibiotic, bacteriophages have evolved over millennia to have the ability to respond according to different environmental conditions so they can maximize their success in terms of the number of viral progenies made. Most phage isolation is carried out from incredibly fruitful sources such as sewage and using exponentially growing bacterial cells. Both aspects however will select for particular types of phages that may not work under the actual conditions needed to treat bacteria during chronic infection. In chronic infection, cells will often be in stationary phase, in nutrient limited conditions and in variable oxygen levels. The bacteria have evolved their own success strategies in these conditions and thus phages have evolved many ways in which to accommodate this. I will give examples from work in our laboratory and that of others that relates to the three ecologically relevant parameters, temperature, oxygen status and growth status. I will show how phages respond to these conditions and show how we have used different genomic and structural approaches to connect these phenotypic observations to a mechanistic understanding of why they are behaving in this way. Such insights should really enhance our ability to develop bacteriophages in an informed way. There are no existing frameworks to contextualize bacteriophage's ecological traits, so we lack an understanding of which of these traits lead to the best therapeutic outcomes. To address this, we have been working on a repurposed ecological framework called the CSR framework, developed to understand plant ecological strategies. I will present this framework and the progress that we are making to better develop a knowledge of ecological strategies within Phage Therapy. I will end my presentation by sharing some recent data on the use of phages on clinically relevant urinary tract infection (UTI) models. The phages used in these studies took many years to identify, characterize and combine to get to the point where we could use them in this preclinical testing. Ultimately, it makes sense that embedding ecology firmly within processes of bacteriophage isolation, development and usage will significantly accelerate the progress of phage development and ensure that the most optimal phages are developed for therapeutic purposes.

gut virome | infant gut mcirobiota | humanized mouse model | vertical transmission | proximity ligation

Gut virome colonization dynamics in a humanized mouse model of early life development

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The human gut microbiota, a dense community of microorganisms residing in the gastrointestinal tract, assembles according to a defined developmental program during the first 1,000 days of life. Many chronic conditions are causally associated with impaired gut microbiome development, including childhood stunting. We developed a humanized mouse model of early life development providing the experimental framework to test phage-driven remodeling of the gut microbiota during this crucial period. After colonizing germ-free adult mice with fecal samples from 2 healthy infants and 1 toddler, mice were bred, and their pups studied until sexual maturity. Regular fecal sampling of breeders and pups allowed us to track colonization dynamics and vertical transmission of bacterial and viral taxa longitudinally. Fecal samples were processed for bacterial and viral metagenomics using a combination of amplicon, shotgun, and proximity-ligation (“Hi-C”) sequencing. Despite robust colonization of bacterial taxa in mice (44%), only 11% of viral operational taxonomic units (vOTUs) colonized these same mice, resulting in virome compositions distinct from those of the human donors. Over 95% of phage-host predictions were concordant at the phylum-, class-, and order-levels when comparing predictions from a recently developed bioinformatic tool to our proximity-ligation dataset. However, these prediction methods diverged significantly at the genus level, where there was only 67% concordance between approaches. Harnessing our multiple sequencing datasets and approaches, we also examined the impact of prophage induction in shaping virome composition in humanized mice, as a large proportion of vOTUs were detected in mice but not the original donor samples.

Chronic Obstructive Pulmonary Disease | COPD | viral airway microbiome | DNA quantification | sputum samples | bacteriophages

Uncovering the Viral Microbiome in COPD Lungs through Metagenomics

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Chronic Obstructive Pulmonary Disease (COPD) encompasses progressive lung conditions, including emphysema and chronic bronchitis. COPD is currently the third major cause of mortality in the United States and in the United Kingdom. Within COPD patient sputum, there are higher-than-average loads of bacterial counts; these are dominated by *Haemophilus influenzae, Streptococcus pneumoniae, Pseudomonas aeruginosa* and *Moraxella catarrhalis*. To date, even with the bacterial community known, very little has been studied or uncovered about the viral airway microbiome. To understand and observe any viral presence within these lungs, what is the best method of DNA quantification? From a sputum sample the removal of eukaryotic and bacterial particles is an easy process, leaving behind any viral fragments which can be extracted. Yet this obscures the presence of any temperate bacteriophages, and possibly any actively bound phage potentially in the community. If the whole sputum sample is extracted for DNA, the correct measurements must be obtained to analyze all present viral data. In this study, we aim to uncover and identify any putative viral contigs (vOTUs) found in previously extracted DNA from whole sputum samples. From 59 sputum samples, 16,671 vOTUs were found. These vOTUs were compared to known vOTUs to find: the complete viral genomes, viral family groups, lytic or temperate life cycles predictions, and the host prediction range. With this new data, a baseline of identification and COPD viral biome understanding can be implemented in future studies.

Session 11: Phage in food and agriculture

Food Safety | Biocontrol | phages

Biocontrol potential of bacteriophages to enhance food safety throughout the food production and processing chain

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In the face of recurrent foodborne outbreaks and product recalls, the need for safer foods remains in the public interest. The extended overuse and misuse of antibiotics and other antibacterial agents in the food industry have further exacerbated the spread of foodborne pathogens with the rise in antimicrobial resistance. This increased demand for safer food products with clean label drives the continuous search for natural alternatives to control the growth of pathogenic bacteria. Bacteriophages have emerged as a legitimate antibacterial alternative with a wide scope of applications which continue to be discovered and refined. From farm to fork, bacteriophages have been shown as a viable option to treat diseases in animals and plants, reduce biofilm formation and contamination in food processing environment, and increase the safety and shelf-life of food products. In this presentation, our research group effort in application of lytic phages to tackle selected bacterial pathogens will be presented. Suggested approaches to overcome the challenges of phage applications as biocontrol tools throughout the food supply chain will also be discussed. This presentation will start with developing a spry dried phage-carrier biopesticide to control *Erwinia amylovora*, the causal agent of fire blight disease in apple and pear. Then, the potential of *Listeria* lytic phage to disperse *Listeria monocytogenes* biofilm will be evaluated in a simulated food processing conditions. Finally, a smart and high throughput approach for developing a phage cocktail to mitigate the risk of *Salmonella* in poultry products will be presented.

phage food safety directed evolution Listeria monocytogenes phage resistance

Overcoming hurdles for phage applications in food safety with directed evolution

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Phage-based products are currently in use in the United States and globally to combat the foodborne pathogen, Listeria monocytogenes. However, their efficacy might be constrained by two critical hurdles. Firstly, the development of phage resistance is predictably selected for under the pressure of lytic phages. Secondly, the complexity of various food matrices may restrict the lytic capabilities of phages. In this presentation, I will explore the potential of directed evolution as a strategy to mitigate these hurdles, particularly in relation to phages infecting *Listeria monocytogenes*. I will specifically highlight how we used directed evolution to select *Listeria* phages capable of countering phage resistance. Additionally, I will share our experiences in selecting a mutant *Listeria* phage demonstrating enhanced binding efficiency in milk conditions through a similar approach.

Agrobacterium phage biocontrol tomato hairy root disease

Back to the roots: phage biocontrol of *Agrobacterium* causing hairy root disease in tomato production

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Agrobacterium biovar 1 is a plant pathogen with the ability to colonize the irrigation system of tomato greenhouses, causing hairy root disease (HRD). Management focuses on hydrogen peroxide to disinfect the nutrient solution, but due to the emergence of resistant strains, its efficacy and sustainability are questioned.

Using a relevant collection of pathogenic *Agrobacterium*, consisting of >10 species, a diverse set of lytic phages, named OLIVR, were isolated from infected greenhouses. A combination of three can infect 85% of the complete collection. All phages remained stable under greenhouse-relevant conditions (pH, peroxide, temperature, nutrient solution). To assess the efficacy of the phages, their ability to disinfect greenhouse nutrient solution inoculated with agrobacteria was tested. Each phage infected their host, but their ability to decrease the bacterial concentration and limit phage resistance development differed. Phage resistant mutants were tested for reduced virulence.

Based on these data, a phage cocktail was selected and tested in small-scale plant experiments (tomato plants in pots) and in a commercial greenhouse setup (240 tomato plants divided over three objects: bacteria only, negative control, phage and bacteria treatment). In the latter setup, the plants were treated four times at the start of the production season with bacteria and/or phages and the plants were followed during the whole growth season (9 months), after which they were scored for hairy root disease symptoms.

The cocktail was shown to significantly reduce disease symptom development. Together, these data show the potential of these phages as tool to tackle HRD.

SciPhage | phage-based therapies | animal health | SalmoFree | poultry Salmonella control | Weissella tructae in aquaculture

SCIPHAGE improving global health with the power of phages: "Advancements in Phage-Based Therapies for Animal Health in Colombia: Focus on Poultry and Aquaculture"

Viviana Clavijo [1*]
1. Sciphage

SciPhage is a pioneering Colombian company that has been leading the development of phage-based therapies in Latin America since 2014. We have made significant advancements in the field of bacteriophages applied to animal health, with a specific focus on poultry and aquaculture. Our research focuses on investigating the benefits and therapeutic potential of bacteriophages in controlling *Salmonella enterica* in poultry and *Weissella tructae* in aquaculture.

One of our products is SalmoFree for poultry, a patented product in Colombia, USA, and Brazil. SalmoFree is a phage cocktail designed to effectively control Salmonella, a pervasive problem in production due to its high mortality rates (ranging from 10% to 80%) in birds. The product has undergone successful validation in broilers, and ongoing validation studies are currently being conducted in layer hens and breeders. This validation process includes a safety study. For this study, we evaluated the effect of phages on 272 laying hens. Two groups were assessed: a control group and a treatment group, each consisting of 6 replicates of 23 hens. The treatment group was given SalmoFree® at weeks 2, 6, 14, 19, and 23 of bird age. In weeks 14, 19, and 23, two doses of SalmoFree® were administered with a three-day interval between them. Swabs and organs were analyzed to isolate Salmonella before each dose using traditional microbiology. The detection of phages was performed before and after the doses in weeks 2, 14, and 23. Additionally, we analyzed eggs to isolate Salmonella and detect phages in weeks 21, 22, and 24. Moreover, histopathology and serology assays were conducted on chickens at 1 day of age and hens at 24 weeks of age. Finally, we recorded production parameters (weight, food consumption, and egg production) for 24 weeks. During the study, no Salmonella was isolated, and no mortality associated with phage consumption was observed. Phages were detected 24 hours after the doses.

Furthermore, we are actively developing a product for aquaculture industry to control *Weissella tructae* in trout. This disease causes mortality in trout when the fish reach a weight higher than 150 g. Currently, research and validation studies are underway, and we anticipate obtaining valuable results. Samples of water and trout organs were collected from the Cundinamarca, Risaralda, and Antioquia providence in Colombia to isolate *Weissella tructae* bacteria and Weissella phages. Infection and one-step curves, host range studies, stability assessments, genome sequencing, and electron microscopy were performed to characterize the phage. We isolated 7 bacteria and purified 12 phages. Out of the 12 phages, 7 have the same host range, and the phages Sp-W001 and Sp-W002 showed better infection efficiency as well as stability in terms of temperature and pH. Based on these results, we propose a phage cocktail consisting of 2 phages to prevent weissellosis in trout in Colombia

Furthermore, SciPhage, as a biotechnology company, has successfully advanced in scaling up the production of its products while complying with relevant regulations. In May, the company inaugurated its production plant, which adheres to good manufacturing practices for animal feed. The setup of the plant posed a significant challenge for the company, as there were no similar plants in Colombia that could serve as a model. Moreover, it is a great milestone for the biotechnology industry in Latin America, as there are few phage production plants in the region. This plant was designed to meet part of Colombia's poultry demand with plans to expand its capacity up to fourfold. The production plant is projected to serve as the scaling and production unit for all portfolio developments of the company, as well as providing services to strategic partners. This work contributes to the understanding and practical application of bacteriophages as a promising alternative in animal health, emphasizing the importance of efficient and scalable production for commercial use.

Session 12: Molecular phage biology II

phage | prophage | Appelmans | directed evolution | Pseudomonas aeruginosa | Paenibacillus larvae | sequencing | genomes

Directed evolution using Appelmans allows for induction and evolution of prophages with expanded host ranges.

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Manipulating the forces of selection can steer phage evolution toward desired ends, such as expanding host range of phages for phage therapy applications. Appelmans method is a passage-based protocol for expanding host range where a mixture of phages are distributed onto hosts—some permissive and some nonpermissive—in separate wells of a plate, incubated, and pooled again after growth before repeating. We sought to test if this method would effectively expand host range of phages that target *Pseudomonas aeruginosa* by passaging three phages (for nine iterations) on five strains of *P. aeruginosa*—three permissive and two nonpermissive. After only two rounds of passaging, host range expanded onto the nonpermissive hosts. Whole genome sequencing of phages isolated from Appelmans allowed for the identification of mutations compared to parent phages, although surprisingly, no mutations were found in known tail fiber genes as anticipated. Sequencing data also revealed that induction and evolution of prophages from two different bacterial hosts used in Appelmans had occurred, and contributed to variation of host range. Similar results were also observed when Appelmans was utilized to evolve phages against *Paenibacillus larvae*, the etiological agent of American Foulbrood disease in honeybees. These unexpected results of prophage induction and evolution during Appelmans demonstrate the advantages of generating complete reference genomes of bacterial host strains intended for directed evolution purposes. It also provides insight into the potential of prophage “contamination” of phage lysates, which could have negative impacts in the case of transduction of harmful bacterial genes, but may also have positive impacts such as the generation of virulent mutants of prophage with improved host ranges.

Microbiology | Molecular Biology | Biochemistry | High throughput | Cell Biology

Biasing Bacteriophage Behaviour

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Bacteriophages have two well characterized life cycles; lytic and lysogenic. Most phages are capable of undergoing both, these are referred to as temperate. The factors that influence a temperate phage's decision of whether to enter the lytic or lysogenic cycle are hotly debated; however, it is clear that the decision has an enormous impact on bacterial communities.

The Hynes' lab has been working on ways to bias the phage lysis/lysogeny decision to better understand the underlying mechanism. Dormant phages are canonically induced out of the bacterial genome in response to the activation of the SOS pathway for DNA repair. This is often metaphorically likened to phages 'abandoning a sinking ship'. Several DNA-damaging antibiotics are known to induce phages. We found combining temperate phages with SOS-inducing antibiotics improved their effectiveness as bactericidal agents by inducing any formed lysogens. Curiously, we found other antibiotics that decreased the frequency of lysogeny by biasing the initial lysis/lysogeny decisions. These recent findings support our hypothesis that this decision is far more complex than simply the activation of the SOS response by DNA damage.

To explore this, we have conducted high-throughput screening of 3921 bioactive compounds exposed to wild type *E. coli* and *E. coli* carrying temperate phage, HK97. 'Hits' are classified as differences in cell density from phage-driven bacterial lysis in the presence of the compound between bacteria with and without dormant phage. We found 37 compounds causing phage mediated bacterial killing. A few compounds were randomly selected and verified in lab through growth curve analysis and measuring end point phage concentration. Verified 'hits' included expected fluroquinolones and beta-lactams, known activators of the SOS response, as well as unexpected compounds such as Prozac, a commonly prescribed SSRI, and berberine, a plant alkaloid used in Aryuvedic medicine. These commonly consumed compounds may be resulting in phage-driven effects through biasing the lysis/lysogeny decision.

We are currently repeating the HTS using a range of concentrations as well as in a bacterial host unable to activate the SOS response, to determine which interactions depend upon that bacterial pathway and which are operating independently. To identify mechanisms involved in non-SOS activating compounds, we will expose phage + compound to an *E. coli* mutant library and identify which host pathways are needed to generate observed effects. This will enable possible identification of new bacterial stress pathways.

Our screening to date has been able to identify phage mediated killing with exposure to novel, commonly consumed, compounds. This is the first systematic approach to identifying compounds influencing the lysis/lysogeny decision and will likely reveal many new mechanisms by which phages can guide their decision-making behaviours. These, in turn, can be used to exploit phages and manipulate bacterial populations to improve the therapeutic application of phages.

Cell morphology | Spbetavirus | Bacillus subtilis | Active lysogeny | Lysogenic conversion

Aberrant *Bacillus subtilis* cell's morphology emerges as consequence of active lysogeny

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Prokaryotic viruses of Spbetavirus genus are temperate bacteriophages targeting *Bacillus subtilis*. As these phages carry genes encoding for bacteriocins, communication systems and regulators of sporulation/germination processes, they substantially alter host phenotype during lysogeny. The impact they exert on the bacterial host is strengthened by their ability of active lysogeny (or regulatory switch mechanism, RS), an alternative lysogenic life cycle characterized by integration into specific bacterial functional genes, resulting in inactivation/reactivation of the target genes by phage integration/excision, respectively. Temperate phages characterized by following this specific life cycle are also named RS-phages. While investigating the impact of RS-phages on *B. subtilis* physiology, we observed a cell morphology change caused by a specific Spbetavirus, resulting in an aberrant spherical shape as opposed to a conventional rod shape showed by the wild type strain. This phenotype manifests in late exponential phase and during sporulation, being stable and heritable. Furthermore, we also discovered that superinfection with a second homologous Spbetavirus restores the conventional rod-shaped morphology, suggesting a phage-phage interplay which results in protecting the host from the cell morphology shifting. We investigated potential differences between the wild type and its spherical-shaped lysogenic version through Transmission Electron Microscopy analysis and Fluorescence Microscopy's time-lapse experiments using various reporter fusion constructs, questioning mainly processes like cell elongation, cell division and sporulation. Moreover, we obtained relevant results investigating the single and double lysogeny influence on phage/s and host fitness through plaque and growth assays, observing variety of plaque morphologies correlated to peculiar growth dynamics. Additionally, the sequencing analysis helped to explore the impact of the double lysogeny on the host genome, in comparison with the single lysogens. Interestingly, the effect on host morphology does not seem unique of a specific prophage, but it is exerted by other closely related phages belonging to the Spbetavirus genus, as we observed while screening our lysogens collection.

Exploring the mechanism behind the host's morphology change by specific Spbetaviruses will increase the current limited knowledge on lysogenic conversion, likewise interference of prophages with fundamental cellular processes. The outcome of this research will bring us closer to understanding the role of prophages in controlling bacterial hosts, highlighting mechanisms still unknown, crucial knowledge to control bacteria for biotechnological and medical applications.

Phage tRNAs Host Resistance Bacteriophages Genetic Material

Phage tRNAs Subvert Host Resistance

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Bacteriophages are an integral component of the evolution of bacteria through population control, spreading of genetic material by horizontal gene transfer, and introducing genetic novelty through the selection for resistance. Bacteriophage-host interactions have been the basis on which many biotechnological tools were discovered, such as restriction enzymes and CRISPR-Cas9. It has been known for several decades that phage T4 encodes tRNAs, but more recently, we have found that about 20% of fully sequenced phage genomes also encode tRNAs. Yet why this is the case remains unknown. The main hypothesis, the Codon Usage Bias Hypothesis (CUBH), suggests that differences between bacteriophage and host in codon choice necessitates that bacteriophage encode their own tRNAs to complement host tRNAs. Here we show that codon usage bias does not explain the presence of tRNAs in phage genomes. Instead, through experiments in T4 and *E. coli*, we show preliminary evidence that tRNAs are involved in host-range by reducing the rate at which spontaneous phage resistance mutants arise. We are currently investigating the mechanistic basis for the control of host-range by tRNAs in this model system.

prophage transduction mobility recombination homology

Genomic mobilisation by rRNA operon recombination – another route of phage transduction

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Multiple rRNA operons in bacteria can facilitate homologous recombination of both horizontally acquired and genomic DNA (Lan & Reeves, 1998; Sato & Miyazaki, 2017). Indeed, homologous recombination between rRNA operons can cause large genomic rearrangements in Salmonella (Helm et al., 2003; Liu & Sanderson, 1998) and E. coli (Hill & Harnish, 1981). Here we show that Staphylococcus aureus rRNA operons also facilitate homologous recombination and that this can promote a novel route of phage mediated transduction, here called recombined transduction. Transduction is the process whereby bacterial DNA is packaged and transferred between cells by phages (Ning et al., 2019). Here we show that large DNA circles formed by homologous recombination of two neighbouring rRNA operons flanking a prophage can be packaged by the phage when induced, leading to recombined transduction at a higher rate than the basal level of generalised transduction. Furthermore, this phenomenon seems to occur more widely, with similarly structured sequences identified in Salmonella. Our results further blur the lines between the core genome and mobile genetic elements and establishes another form of phage mediated transduction, recombined transduction.

Tequintavirus | Escherichia coli | Shiga toxin | host recognition | tail genes

Characterizing the role of tail genes in host recognition of *Tequintavirus* with anti-Shiga toxin-producing *Escherichia coli* activity

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Background: Shiga toxin-producing *Escherichia coli* (STEC) continually challenge the food safety system worldwide. Bacteriophages demonstrate great potential as a biocontrol agent for STEC in the food supply chain. Naturally occurring phages are ubiquitous in cattle and their environment, regulating the diversity of STEC via unknown mechanisms. **Hypothesis:** We hypothesized that endogenous phages within the same genera undergo alterations in their tail genes to enhance their ability to infect various serogroups of STEC. **Objective:** This study characterized the lytic activity and genomics of six STEC-infecting phages isolated from commercial feedlots in Alberta and elucidated the function of tail proteins in host recognition and attachment. **Methods and Results**: Overall, the six phages (e.g. AHF125, AXO103A/B/C, AXO45B, and AXO26A) exhibited broad host range and strong lytic activity against STEC serogroups O26, O45, O103, O145 and O157 in the laboratory. The estimated adsorption rate of these phages ranged from 5.2×10^10 to 2.2×10^9 ml/min. Taxonomically, they belonged to the *Tequintavirus* genus of the Markadamsvirinae subfamily, containing 106 – 109 kb dsDNA (155 – 173 coding sequences, 22-24 tRNAs). Comparative genomics revealed that the genomes of the phages had a similarity of 86–89%. However, their L-shaped tail fibers (LTFs) and receptor binding proteins (RBP), which are known to be responsible for host attachment varied (e.g. as low as 24% identity of LtfA of phage AXO45B to that of reference phage AKFV33) at the amino acid level. Unlike the typical Ltf structure of *Tequinatavirus*, Ltfs of STEC phages consisted of 2–3 gene products, namely LtfA and LtfB, with or without a hypothetical protein orf between these gene products. To determine the function of these unique Ltf and RBP toward host adsorption, Ltfs and RBP of O157-infecting phage were cloned, expressed, and purified for use in a phage adsorption inhibition assay. In the presence of LtfA (≥0.05 mg/ml) and orf136 (≥0.1 mg/ml), 66 – 82% of phage failed to attach, a higher proportion ($p < 0.001$) than that of the protein-free control (~ 0.2 % unabsorbed phages after 15 min). This observation suggests that LtfA and orf136 are bound to the same bacterial receptors as the parental phages, preventing them from initiating adsorption. **Conclusions:** Environmental *Tequintavirus* was efficacious against multiple clinically important STEC serogroups. Variations of genes encoding for Ltfs and RBP from *Tequintavirus* may be responsible for STEC infectivity.

Keywords: Shiga toxin-producing *Escherichia coli*, bacteriophages, Host range, phage adsorption, *Tequintavirus*, L-shaped tail fiber, receptor binding protein.

adenosine diphosphate ribosylation | ARTs | RNAylation | ribosomal proteins | T4 phages | E. coli

Discovery of a molecular glue to link RNAs with proteins in E. coli during T4 phage infection

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The mechanisms by which viruses hijack their host's genetic machinery are of enormous current interest. One mechanism is adenosine diphosphate (ADP) ribosylation, where ADP-ribosyltransferases (ARTs) transfer an ADP-ribose fragment from the ubiquitous coenzyme nicotinamide adenine dinucleotide (NAD) to acceptor proteins. Here, we report that a bacteriophage T4 ART ModB surprisingly accept not only NAD but also NAD-capped-RNA as substrate, thereby covalently linking entire RNA chains to acceptor proteins in vitro and in vivo. We term this reaction an "RNAylation". *Salmonella* ModB specifically RNAylates ribosomal proteins rS1 and rL2 at defined arginine residues, and a specific group of E. coli and T4 phage RNAs is linked to rS1 in vivo. T4 phages that express an inactive mutant of ModB show a decreased burst size and slowed lysis of E. coli. Our findings reveal a distinct biological role of NAD-RNA, namely activation of the RNA for enzymatic transfer to proteins. The attachment of specific RNAs to ribosomal proteins might provide a strategy for the phage to modulate the host's translation machinery. This work exemplifies the first direct connection between RNA modification and post-translational protein modification. As ARTs play important roles far beyond viral infections, RNAylation may have far-reaching implications in the cellular context. Moreover, the discovery of the RNAylation might provide a starting point for the application of RNAylated-proteins as next-generation RNA therapeutics.

Poster Session

Klebsiella pneumoniae | Lytic bacteriophages | Receptor Binding Proteins | Capsules | Structural Modelling

Using Structural Modelling and Sequence Analysis to Study Receptor Binding Protein Diversity and Specificity in Lytic Phages of *Klebsiella pneumoniae*

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A successful phage infection usually depends on an interaction between a receptor-binding protein (RBP) and a bacterial receptor. In *Klebsiella pneumoniae*, some phages use depolymerase-containing RBPs to degrade capsule polysaccharides. Previous studies have shown that phage RBPs in *Klebsiella* are highly diverse and very specific towards the bacterial sugar. However relatively little is known about the diversity of these RBPs at the structural level. Here we hypothesised that structural modelling using AlphaFold2 can improve our understanding of the nature of RBP-sugar specificity by identifying protein folds associated with recognition of specific polysaccharides.

To address it, we constructed a dataset of 36 GenBank Phage RBPs specific to 23 different capsular serotypes in *K. pneumoniae* (12 serotypes have more than one RBP representative) and whose specificities have been experimentally validated via production of recombinant proteins. We generated homotrimeric structural predictions of all RBPs and, where possible, identified three domains manually using PyMol: N-terminal domain, a central (enzymatic) domain and a C-terminal domain. We then compared all sequences, structures and their domains to each other using BLAST, US-align and Foldseek. We assessed structural similarity between protein pairs based on TM-score (TM-score<0.5 unrelated, TM-score≥0.5 related, TM-score>0.9 structurally identical).

Our results point to several observations. First, RBPs are highly diverse genetically. We found that while only 28% of RBP pairs had detectable similarity at the sequence level, 73% of them were related on the structural level. This suggests that many related RBPs would not be detectable at the sequence level.

Second, structural modelling can help predict RBP-sugar specificity. Specifically, RBPs specific to the same capsule types were structurally more similar to each other than RBPs without confirmed specificity to the same capsule (Wilcoxon-rank test, average TM-score = 0.77 vs 0.55, $p<10^{-4}$). This association was even stronger when the comparison was carried out at the level of central-domains (TM-score 0.89 vs 0.72, $p<10^{-5}$) and C-terminal domains (TM-score 0.82 vs 0.34, $p<10^{-5}$), both of which are known to confer host-specificity. No significant association was observed at the level of N-terminal domain ($p>0.5$).

Third, structural modelling can help identify potentially interesting biological and biochemical properties of RBP-capsule interactions. One example were two unrelated structures (TM-score=0.31) of RBPs specific to K47. We found that these structures had been shown to be specific to different subsets of serotype K47 *K. pneumoniae* strains, suggesting potential differences in capsular sugar compositions. Another example were near-identical structures of RBPs specific to K11 and K13/K2 (TM-score=0.87). These capsule types were found to have α/β-D-Glcp-(1→3) residue in common, suggesting that it might be targeted by the depolymerase.

Finally, we saw that phage morphotype was a significant but an imperfect predictor of N-terminal diversity. While structural similarity of N-terminal domains was greater amongst phages assigned to the same morphotype (average TM-score = 0.58 vs 0.44, $p<10^{-5}$), we found multiple examples of structurally identical N-terminals belonging to phages of different morphotypes.

Overall, our results show the importance of applying structural modelling and sequence analysis to study the diversity of RBPs and to understand the evolutionary processes leading to capsular specificity.

Haemophilus somni | Pasteurella multocida | Bacterial Respiratory Disease | Cattle | Pneumonia

Bacteriophage Lysis of Bacterial Pathogens of Calf Pneumonia

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Haemophilus somni and *Pasteurella multocida* are bacterial pathogens causing respiratory disease in cattle and pneumonia in calves. These two organisms were recently isolated from a case of pneumonia in calves. The disease causes high mortality due to the immunocompromised state of the calves. During the outbreak, the calves examined showed clinical signs of severe bronchopneumonia, including anorexia, coughing, nasal discharge, dyspnea, diarrhea, distension of the neck, lethargy, recumbency, lameness preceding collapse, and high mortality was recorded. Treatment with ceftiofur a drug of choice showed a seeming resistance with a continued occurrences of mortality in the herd. These two organisms were used as host, and were completely lysed by a bacteriophage from a tannery waste water. This phage when fully harnessed could be a possible control of Bovine Respiratory Disease (BRD) in cattle or Pneumonia in calves.

bacteriophages | phage therapy | antibiotic resistance | Extraintestinal pathogenic E. coli

The Coevolutionary Arms Race between Bacteria and Therapeutic Phages: Implications for Phage Therapy

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The crisis of MDR pathogens has led to rare use of bacteriophages (phages) as a therapeutic. However, bacterial resistance to phage threatens to undermine phage therapy. While well explored in laboratory settings, there is little research regarding the development of phage resistance under therapeutic conditions. In this study, we explore the coevolutionary arms race between bacteria and phages, using clinically-derived isolates of Extraintestinal pathogenic *E. coli* (ExPEC) and well-studied phages (primarily ϕHP3) which have been used therapeutically for patients with infections recalcitrant to antibiotics.

The development of phage resistance (“resisters”) by the bacteria was studied using two parallel methods: a plate-based method, and a murine model of bacteremia. Resisters isolated from both methods were assessed for viability in simulated host environments. Most murine-derived resisters were attenuated in human urine, and all resisters failed to survive in human blood. Strikingly, tested resisters further failed to cause severe disease in the very model of bacteremia from which they were isolated. Sequencing revealed highly conserved mechanisms of resistance, suggesting ST131 isolates preferentially develop resistance to ϕHP3 through changes to its receptors. Using the Appelman’s method or a continuous bioreactor that we designed, we directed the evolution of ϕHP3. The resulting two phages, ϕHP3.1 and ϕHP3.2, were remarkably similar to parental ϕHP3.

To test the efficacy of phage cocktails to prevent phage resistance, we constructed four phage preparations (three cocktails and a singular phage). Measuring bacterial growth and regrowth revealed that, as is currently believed, phage cocktails are better at preventing the development of phage resistance than single phage preparations. Interestingly, the cocktails containing evolved phages were more efficient at preventing resistance the wild type cocktail. This finding underscores the importance of anticipating resistance mechanisms and adapting phages to common resisters.

In this study, we found that ExPEC resistance to phage is associated with attenuation in host microenvironments and systemic infection. We found that phage-resistant pathogenic *E. coli* readily develop, but are often attenuated. Further, cocktails of phages can decrease the likelihood of resister development and, likely, impose greater fitness losses in breakthrough resisters. This work lays important groundwork for the use of phage therapeutics while countering bacterial resilience.

phage therapy | cystic fibrosis | pseudomonas aeruginosa

A study evaluating phage therapy in cystic fibrosis subjects with *Pseudomonas aeruginosa* Infection.

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Introduction: Cystic fibrosis (CF) is a genetic disease that sensitizes the lungs to life threatening bacterial infections and thus requires chronic antibiotic use. *Pseudomonas aeruginosa* (*PsA*) is a notorious antibiotic-resistant pathogen associated with morbidity and mortality of CF patients. Bacteriophage therapy offers a novel alternative or adjunctive option to antibiotics in chronic *PsA* infections.

Objective: The objective of this phase 1b/2a study was to assess the safety and tolerability of nebulized phage (BX004) and its effect on sputum *PsA* burden.

Methodology: Nine CF patients chronically infected with *PsA* were enrolled in Part 1 of an ongoing phase 1b/2a double-blinded placebo-controlled clinical trial. Seven patients received ascending doses of BX004 whereas two patients received placebo. All patients received their standard-of-care antibiotic treatment during the trial.

Results: BX004 was safe and well-tolerated. Furthermore, patients treated with phage showed an average log 1.42 CFU/g reduction in *PsA* sputum density compared to baseline, whereas the reduction from baseline in the placebo group was log 0.28 CFU/g.

Conclusions: These data pave the way toward the establishment of novel phage-based inhaled therapeutics for chronic PsA infections in CF.

Helicobacter pylori | antibiotic resistance | bacteriophages | prophages | phage therapy

Phage Therapy: A New Era in the *Helicobacter pylori* treatment?

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Abstract: *Helicobacter pylori* infection which affects more than half of the world population, is considered the most important risk factor in the pathogenesis of gastric cancer [1, 2], and its eradication might confer long-term protection against gastric adenocarcinoma in high-risk populations [3]. The treatment usually combines antibiotics and an acid-reducing proton pump inhibitor [4]. Nevertheless, increasing antibiotic resistance and ineffective treatments led the World Health Organization to designate clarithromycin-resistant *H. pylori* as a high-priority bacterium for antibiotic research and development [5]. These findings call for alternative strategies to control *H. pylori*. Phages, viruses that infect bacteria, show effectiveness in the treatment of pathogenic bacteria [6], however very little is known about *H. pylori* phages [7].

Due to the difficulties in isolating *H. pylori* strictly lytic phages and envisaging the possibility of using temperate phages to control *H. pylori* infections, we develop a method to identify prophages in *H. pylori* genomes and to induce their lytic cycle. We used a collection of 74 *H. pylori*-clinical strains to screen for prophage genes based on PCR profiling. Strains encoding putative prophages were further sequenced. Subsequently, after genome analysis, prophage induction was attempted in strains with intact prophages, using UV radiation and mitomycin C. The isolated phages were further characterized.

Based on the results of the PCR-detection method, 14 strains, with a high probability of having prophages in their genome were sequenced, resulting in a total of 12 prophages identified *in silico*. The developed methodology increased the efficiency of identifying strains with complete prophages to 83.3 %. Induction strategies against strains with positive-prophage sequences resulted in the release of three new phages with a podovirus-like structure. They present a genome length range between 29 705 and 31 162 bp with a G+C content of around 37 %. No tRNAs or antibiotic-resistance genes were identified. Interestingly, the three phages were relatively stable from pH 3 to 11 and at 37 ◦C, suggesting that they are adapted to the human stomach environment. Furthermore, two phages had the capability to suppress *H. pylori* population levels for up to 24 h post-infection at multiplicities of infection of 0.01, 0.1, and 1. The phylogenomic tree conclusively demonstrated that these new phages are phylogenetically related to all other reported *H. pylori* phages because, with one exception, they all belong to the same family and genus, even though all of them have different geographical origins.

Overall, our findings reveal a novel area for future investigations representing a significant contribution to the knowledge of *H. pylori* phages and providing valuable insights into their potential use in phage therapy.

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Pseudomonas aeruginosa | prophages | bioinformatic analysis | ESKAPE pathogens

Pseudomonas aeruginosa prophages: a new and simplified bioinformatic analysis

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ESKAPE pathogens are a global threat caused mainly by the misuse of antibiotics and, according to the World Health Organization (WHO), should be targeted with high priority. Horizontal gene transfer (HGT), which allows bacterial cells to exchange genetic information, leads to the widespread of antibiotic resistance genes (ARGs) and consequently the emergence of antibiotic-resistant variants. Transduction is an important mechanism of HGT and therefore understanding and controlling transduction events becomes crucial. In a previous work, we showed that prophages have a high impact on *Acinetobacter baumannii* genomic context, by encoding different virulence and fitness-related genes. In this study, we developed a pipeline to simplify the analyses of prophages from a set of genomes of *Pseudomonas aeruginosa* clinical isolates, by conjugating different publicly available bioinformatic tools.

For the analysis, 449 genomes from *P. aeruginosa* clinical isolates were submitted to PHASTEST to search for prophage regions. The detected prophage genomes were sorted by their type (intact, questionable, incomplete), and their size and frequency were analyzed. Intact prophages were selected for further analysis consisting of predicting their taxonomic family, searching for ARGs, and looking for other virulence/fitness-related genes

Results showed that prophages are prevalent among these *P. aeruginosa* isolates. The average number of intact prophages per genome was three, but some genomes carried up to ten intact prophages. Despite the analyzed genomes being organized in several contigs, surprisingly there is a low prevalence of incomplete prophages. We also found a worrying number of ARGs, such as *catB* and bcr_1, as well as other virulence factors related to alginate, pili, and secretion systems, confirming these intact prophages' potential role in virulence spread. Prophage morphology was also predicted with myovirus being the most prevalent (>70%) followed by siphovirus (<30%), no podovirus was found.

In conclusion, with our pipeline, we showed that prophages are widespread among *P. aeruginosa* clinical isolates, with a significant proportion of them being potentially inducible. Among the genes encoded, we found a significant portion of virulence genes, proving the importance these entities have in bacterial genetic evolution. More studies about prophages should be performed using large clinical isolates in order to understand the current status of these pathogens and monitor bacterial genes transfer among them.

phage K | Staphylococcus aureus | temperature-sensitive | USA300 | polyamines | loss-of-function mutation

Loss-of-function Mutations in Phage K gp102 Lead to Improved Antibacterial Activity against USA300 MRSA Growing at 37°C or Lacking the *potABCD* Operon

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Among *Staphylococcus aureus* infections, the USA300 lineage is a frequent cause of invasive disease. We observed that phage K, a model *S. aureus* myophage, exhibits temperature-sensitive growth on USA300 strains, with the wild-type phage providing poorer growth suppression in broth and forming smaller and fainter plaques at 37°C vs. 30°C. We isolated 65 mutants of phage K that had improved plaquing characteristics at 37°C when compared to the parental phage. In all 65 mutants, this phenotype was attributable to loss-of-function (LoF) mutations in *gp102*, which encodes a protein of unknown function that has homologs only among the *Herelleviridae* (SPO1-like myophages infecting gram-positive bacteria).

Additional experiments with representative mutants consistently showed that the temperature-sensitive plaque phenotype was specific to USA300 MRSA strains and that Gp102 disruption was correlated with improved suppression of bacterial growth in broth and improved antibacterial activity in a mouse model of upper respiratory tract infection. The same genotype and in vitro phenotypes could be replicated in close relatives of phage K. Gp102 disruption did not have a detectable effect on adsorption but did delay cell culture lysis relative to wild-type under permissive infection conditions, suggesting that *gp102* conservation might be maintained by selective pressure for more rapid replication.

Molecular modeling of Gp102 predicts a protein with two helix-turn-helix domains that displays some similarity to DNA-binding proteins such as transcription factors. While its function remains unclear, *gp102* is a conserved gene that is important to the infection process of *Kayvirus* phages, and it appears that the manner in which USA300 strains defend against them at 37°C can be overcome by *gp102* LoF mutations. Additionally, deleting the polyamine uptake system (*potABCD*) from USA300 strains reduces the activity of phage K at both 37°C and 30°C; Gp102 disruption overcomes this phage growth restriction as well.

antibiotic resistance; bacteriophages; phage-encoded enzymes

Cloning and Characterization of a Thermostable Endolysin LyZC1 as a Potential Therapeutic Agent

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Research objectives:

The challenge of antibiotic resistance has gained much attention in recent years due to the rapid emergence of resistant bacteria infecting humans and risking industries. Thus, alternatives to antibiotics are being actively searched for. In this regard, bacteriophages and their enzymes, such as endolysins, are a very attractive alternative. Endolysins are the lytic enzymes produced during the late phase of the phage lytic cycle.

Methodology and Results:

Here, we cloned, expressed, and purified LysZC1 endolysin from *Pseudomonas* phage ZCPS1. The structural alignment, molecular dynamic simulation, and CD studies suggested LysZC1 to be majorly helical, which is highly similar to various phage-encoded lysozymes with glycoside hydrolase activity. Our endpoint turbidity reduction assay displayed the lytic activity against various Gram-positive and Gram-negative pathogens. Although in synergism with EDTA, LysZC1 demonstrated significant activity against Gram-negative pathogens, it demonstrated the highest activity against Bacillus cereus. Moreover, LysZC1 reduced the numbers of logarithmic-phase *B. cereu*s by more than 2 log10 CFU/mL in 1 h and acted on the stationary phase culture. Remarkably, LysZC1 presented exceptional thermal stability, pH tolerance, and storage conditions, as it maintained the antibacterial activity against its host after nearly one year of storage at 4 °C and after being heated at temperatures as high as 100 °C for 10 min.

Conclusions:

Our data suggest that LysZC1 is a potential candidate as a therapeutic agent against bacterial infection and an antibacterial bio-control tool in food preservation technology.

Gram negative | Klebsiella pneumoniae | Siphovirus | Multi-drug resistance (MDR) | Bacteriophage | Phage therapy

Morphological, biological, and genomic characterization of *Klebsiella pneumoniae* phage vB_Kpn_ZC2

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Background: Bacteriophages (phages) are an extremely promising alternative to conventional antibiotic therapies, particularly when combating bacteria that have developed resistance to multiple antibiotics. Klebsiella pneumoniae is recognized as an opportunistic pathogen capable of causing potentially fatal infections. Thus, this study aims at the characterization of a novel isolated phage vB_Kpn_ZC2 (ZCKP2, for short).

Methods: Using the clinical isolate KP/08 as a host strain, the phage ZCKP2 was isolated from sewage water. The isolated bacteriophage was purified, amplified, and subjected to various tests, including Pulse-Field Gel Electrophoresis (PFGE) to determine its molecular weight, transmission electron microscopy to examine its structure, analysis of its antibacterial activity against a range of *Klebsiella pneumoniae* hosts, stability studies, and whole genome sequencing.

Results: Phage ZCKP2 has been categorized as a siphovirus based on the results of Transmission Electron Microscopy. The phage genome size was estimated to be 48.2 kbp using Pulsed Field Gel Electrophoresis and phage sequencing. Additionally, the absence of genes related to lysogeny, antibiotic resistance, and virulence in the annotated genome suggests that Phage ZCKP2 is safe for therapeutic use. Based on the genome-based taxonomic analysis, Phage ZCKP2 belongs to a novel family that has not yet been formally classified.

Furthermore, Phage ZCKP2 was found to remain stable at different temperatures and pH ranges (-20 − 70 °C and pH 4 – 9). In terms of antibacterial activity, Phage ZCKP2 consistently produced clear zones on KP/08 bacteria and other hosts and effectively killed bacteria over time at different MOIs (0.1, 1, and 10). Additionally, the genome annotation predicted the presence of antibacterial lytic enzymes. The predicted topology of class II holins in some putative proteins with dual transmembrane domains indicates their significant contribution to antibacterial activity. The characterization of Phage ZCKP2 demonstrates its safety and effectiveness against multidrug-resistant *K. pneumoniae*, thus making it a strong candidate for further clinical applications in vivo and phage therapy.

phages | genome engineering | short DNA repeats | bacterial hosts | CRISPR-Cas

Harnessing short DNA repeats for genome engineering of phages

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Phages are ubiquitous, highly diverse, and can infect a variety of bacterial hosts in several ecological niches. To successfully infect their hosts, phages must develop strategies to escape bacterial defense mechanisms or barriers for infection. Interestingly, we recently noticed when studying *Pseudomonas* phage Ab09 that its genome contains several short DNA repeats, and these repeats could mediate recombination when the viral genome is cut by a CRISPR-Cas system. Additional bioinformatics analyses revealed that these short DNA repeats are in fact highly abundant in phage genomes. Here, we investigated whether we could exploit these short DNA fragments for genome engineering purposes using CRISPR-Cas9.

We aimed to modify four virulent phage genomes infecting various bacterial species, such as *E. coli*, *Salmonella*, *Pseudomonas,* and *Streptococcus*. For *E. coli* phage T7 and *Salmonella* phage Felix O1, we used pCas9 that contained the heterogenous CRISPR-Cas9 system from *Streptococcus pyogenes*. For the genome editing of phage 2972, we used the endogenous CRISPR-Cas system of its host *Streptococcus thermophilus*. To modify the genome of virulent *Pseudomonas* phage Ab09, we first adapted a plasmid containing an heterogenous CRISPR-Cas9 system from *Neisseria meningitidis* (NmeCas9) by adding the pRO1600 origin of replication and the gentamicin resistance gene, generating pNmeCas9P.

By using these heterogenous or endogenous CRISPR-Cas systems, we spacer-targeted specific phage genes that were naturally flanked by short DNA repeats to mediate recombination in the above four phage genomes. Using mostly 10-11 bp repeats, we successfully generated knockouts of three genes (*orf09*, *orf10* and *orf14*) or two genomic regions (*orf06-orf10* and *orf80-orf83*) of *Pseudomonas* phage Ab09. For *Streptococcus* phage 2972, we targeted the gene coding for receptor binding protein as well as *orf41*. In T7, we targeted genes 0.3-0.4, 1.1-1.2, 1.4-1.5, and 1.5 alone and generated deletions in all those genes. Interestingly, in coliphage T7 genome, the deletion of the genes 1.4 and 1.5 was mediated by repeats of 7 bp. For *Salmonella* Felix O1, which had repeats up to 57 bp, we were able to generate deletions in *orf21*, *orf22,* and *orf19-orf20*.

In conclusion, targeting specific phage genes with the CRISPR-Cas9 technology led to viral genome recombination mediated by short DNA repeats, and the generation of several knockout phage mutants. Compared to the traditional strategy using CRISPR-Cas9 for phage genome engineering, which includes cloning of both spacer and repair template, this approach described above reduces considerably the time required to generate knockouts. In terms of phage biology, our findings also raise questions on the importance of short DNA repeats in phage evolution.

antirepressor bacteriophage converting phage shiga toxin STEC Stx-phage

Characterization of the Contributions a Detoxified Stx-Prophage Makes to the Fitness of Its Bacterial Host

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Stx-phages are responsible for the horizontal transmission of Shiga toxin-encoding genes between bacteria, and can enter the lysogenic cycle in their host cells before being induced into lytic replication cycle. The size of most Stx-phage genomes is ~50% > λ phage genome. Due to the ability of prophages to carry and introduce additional genetic material to the host cell, Stx-phages can confer traits on their bacterial lysogen. This research focuses on identifying the function of a hypothetical gene, vb_24B_13c, from Stx-phages Φ24B [GenBank: HM 208303] towards the fitness of its bacterial host. In order to ascertain the function of it, multiple approaches including RNA-seq, NanoString datasets, bioinformatics analyses, and motility tests were used to compare phenotypes of various strains: Naïve (Escherichia coli MC1061), lysogen (MC1061/ Φ24B::Cat), and naïve carrying an arabinose inducible plasmid (MC1061 pBAD_13c).

Motility tests were conducted on semi-solid agar plates at different temperatures. With one drop of bacterial culture on the centre, plates were incubated for 12 hours at 37°C or over 24 hours at 25°C or 30°C. Results showed that motility of MC1061/Φ24B::Cat (m = 6.85 cm) was enhanced compared to MC1061 naïve cell (5.0 cm), at both 37°C and 30°C, suggesting prophage Φ24B contributes to the motility of its host. Similarly, the motility of MC1061 pBAD_13c induced by 0.5%, 1.0% or 2.0% arabinose (m > 9.0 cm) was enhanced at 37°C compared to the naïve cell, indicating prophage gene vb_24B_13c can contribute to the motility of naïve cells even without the presence of other prophage genes. However, there was no enhanced motility of MC1061 pBAD_13c at 30°C with arabinose, suggesting that temperature is a factor that affects the host's motility.

RNA-seq data, as validated by an amylase assay using DNS Reducing Sugar Reagent, showed that α-amylase activity is upregulated in the lysogen. Moreover, NanoString data revealed that overexpression of prophage repressor gene, cI, could potentially inhibit the expression of other prophage genes, including vb_24B_13c, res and stk. This indicates that the lysogen's expression of cI is not high enough to repress these prophage genes.

In conclusion, the expression of prophage vb_24B_13c can enhance the motility of its host cell by upregulating genes involved in flagellar synthesis and rotation, and it is able to inhibit the expression of some its downstream genes, such as res and stk. Moreover, these results confirm that the prophage plays a significant role in reprogramming the metabolic function of the host, indicating a profound influence on the host's phenotype.

bacteriophage | base modifications | Oxford Nanopore sequencing | Mass Spectrometry

The search for unique phage DNA in a fight against deadly bacterial infections

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In 2019, there were 4.95 million deaths from antibiotic-resistant bacteria. Identifying bacteriophages (phages) that have base-pair (base) modifications which can evade bacterial defenses, such as restriction enzymes, can help researchers select the best phages to treat bacterial infections in humans, livestock, and agriculture, as well as impact ocean nutrient cycles to combat climate change. We are developing a framework for discovering new base modifications in phages and identifying their function within the phage life cycle.

The DNA of eukaryotes, prokaryotes, and their viruses can be modified by adding methyl groups to their nucleotides which can regulate gene expression. Phage DNA modifications are more diverse, for example, adding amino acids, polyamines, monosaccharides, disaccharides, and methyl groups. These modifications can regulate gene expression, assist in recognizing self-DNA and assist in DNA packaging. It can also protect DNA from degradation by bacteria restriction enzymes (REs), CRISPR-Cas, and environmental damage. Many of these DNA modifications are yet to be discovered.
Most DNA sequencing technologies require PCR amplification which results in the loss of base modifications before sequencing. However, the DNA sequence they provide can be used to obtain a virtual restriction enzyme digest (virtual digest). The proposed framework will use virtual digests to help identify DNA modifications using the following steps.

Step one: screen phage genomes for DNA modifications by comparing phage DNA RE digests to their virtual DNA RE digests. For example, the phages Kharcho and Ottawa show a blockage in some RE digests. In contrast, their virtual RE digest reveals that the DNA should be cut. This inability of the restriction enzyme to cut the phage DNA may indicate a DNA modification in the phage genome.

Step two: Oxford Nanopore Technology (ONT) DNA Sequencing can reveal the presence of base modifications. We are using ONT DNA sequencing to sequence the raw phage DNA in order to identify known base modifications already in the nanopore algorithms or reveal an unknown base modification.

Step three: if the nanopore sequencer detects an unknown base-pair modification, mass spectrometry will be used to identify the unknown modification. New modifications will be added to the nanopore algorithm to recognize the newly identified base modification in other phages. Expanding these algorithms will speed up this workflow, allowing us to use ONT to quickly identify phages with specific DNA modifications without needing Mass Spectrometry or DNA digests.

Step four: Following the discovery of novel base modifications, I will focus on identifying the genes within the phage genome required for adding the modification using a candidate approach. Candidates will be mutated using reverse genetic strategies to test if their mutation impacts the base modification and if the modification is required for phage viability and evasion of host defence systems. The genes involved in base modification will be inserted into phages which lack those genes to see if they can gain the ability to evade bacterial defenses.

gut microbiome | bacteriophages | lysogenic cycle | phage lytic cycle | DNA damage | human health

One size does not fit all: examining lytic/lysogenic decision-making in commensal *Escherichia coli* prophages

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The human gastrointestinal (GI) tract is colonized by a dense consortium of microorganisms, including bacteria, viruses, archaea, and fungi, which together make up the gut microbiome. While the bacterial component of the microbiome has been studied for decades, providing a wealth of information on how this community impacts human health and disease, other community members including viruses are only beginning to gain attention. The viral component of the gut microbiome (or the virome) is primarily composed of bacteriophages (phages) and is highly individualized, but stable over time. Preliminary studies have found shifts in virome composition during various diseases such as inflammatory bowel disease, but whether these virome shifts play a causal role in disease development or are consequences of disease remains unclear. Whether by bacterial lysis during the phage lytic cycle, or by altering bacterial phenotypes during the lysogenic cycle, it is clear that phages have the potential to alter both the composition and function of the gut microbiome community in diverse ways; distinguishing the effect of these two very different lifestyles and how they are determined is important to our understanding of what roles phages might play in the gut microbiome.

Most bacteria found in the mouse gut contain at least one prophage, underscoring the importance of studying temperate phages in the context of the GI tract. Interestingly, it has also been found that rates of lysogeny vary by bacterial taxa, and that all sequenced *Pseudomonadota* were lysogens, which differed from *Bacteroidota* where only about 20% of this phylum were identified as lysogens. This suggests that lysogeny may be more inherent to the lifestyle and niche of *Pseudomonadota* in the gut, as compared to other taxa. It is not well understood, however, whether these temperate phages are undergoing the productive lytic cycle or the dormant lysogenic cycle in the GI tract. Studies using germ-free mice have found high rates of prophage induction in the GI tract, so much so that the ability of the lysogen to colonize the gut was impaired. On the other hand, a recent study following the fecal virome of one individual for over two years found that prophage induction was steadily maintained at a low rate, perhaps explaining the previously observed stability of the human gut virome. The molecular genetics of the lytic/lysogenic switch have been extensively characterized in model phages such as Lambda, revealing how phages integrate their regulatory circuitry with the status of their host. Notably however, these studies have primarily been done in laboratory-adapted bacterial strains and under laboratory conditions, calling into question whether these mechanisms are relevant *in vivo*, or if additional regulatory processes might control prophage induction in the GI tract.

To begin to address these questions, we previously isolated and characterized a novel temperate phage Kapi1, which infects a strain of commensal *Escherichia coli* MP1 isolated from a healthy mouse. Using luminescent reporter assays to monitor the activity of the Kapi1 *CI* promoter (the master repressor of the phage lytic cycle), we found that Kapi1 favors lysogeny in simulated intestinal fluid (SIF), compared to standard LB growth media. These results were further supported by *in vivo* studies of lysogen stability during colonization of specific pathogen-free (SPF) mice; approximately 85% of fecal MP1 shed from SPF mice retained the Kapi1 prophage and were stable at these levels for 28 days, suggesting that lysogeny with Kapi1 is favored in the GI environment.

It was unexpected that Kapi1 favored lysogeny in SIF, as this media contains bile salts which are known to cause DNA damage, and digestive enzymes which would presumably be stressful for the bacterial cell. Accordingly, we noted a slight induction of *recA-lux* reporters and a minor growth defect of Δ*recA* mutants in SIF, indicating certain levels of DNA damage. The fact that Kapi1 favors lysogeny despite evidence of DNA damage in SIF is at-odds with the classical phage induction paradigm, where DNA damage leads to an activation of RecA to RecA*, which mediates proteolysis of CI, resulting in de-repression of lytic genes and entrance into the lytic cycle. Western blots comparing the stability of Kapi1 and Lambda CI-6xHis in cells treated with the genotoxic agent mitomycin C showed that in contrast to Lambda CI, there was no detectable degradation of Kapi1 CI. This suggests that Kapi1 may instead use phage-encoded antirepressors for its induction, as has been shown for other phages. Antirepressors bind to CI and inhibit its DNA-binding activity, rather than degrading CI altogether; still, this induction mechanism would not explain lysogeny being promoted in DNA-damaging conditions, as antirepressor synthesis is typically directly regulated by LexA, which itself is regulated by RecA*. Interestingly, we found that LexA-6xHis strongly accumulated during bacterial growth in SIF, and that the levels of LexA-6xHis were identical between wild-type and Δ*recA* mutants in SIF, indicating that it is not getting cleaved by RecA* as it normally does during DNA damage. We therefore hypothesized that an unidentified protein is induced during growth in SIF which alters the normal SOS cascade, resulting in increased repression of Kapi1 beyond basal levels observed in LB.

Moving forward, we will first confirm whether LexA accumulation in SIF occurs in isogenic non-lysogens as it does in Kapi1 lysogens, to determine whether this putative SOS-modulating factor is host- or phage-encoded. RNAseq will be subsequently performed to identify genes upregulated during bacterial growth in SIF and interrogate the general status of the SOS regulon, to determine what step in the pathway is being modulated. Overall, this project will significantly contribute to our fundamental understanding of phage molecular biology, as reports of lysogeny being actively promoted in SOS-inducing conditions are extremely rare. Further, understanding these mechanisms may provide insight into the temporal stability of the human gut virome. Increasing our fundamental knowledge of the organisms that inhabit humans will have important future implications in understanding how the microbiome as a whole (including phages) contributes to health and disease.

Cellulophaga phage | DNA amplification | thymidine/uridine modification

Challenges in amplifying *Cellulophaga* phage phi40:2 DNA revealed a unique thymidine/uridine modification.

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Cellulophaga phage phi40:2 is a Baltic Sea phage that infects *Cellulophaga baltica* and displays a large Myovirus morphology. However, sequencing its genome has proven challenging using classical methods. Previous attempts utilizing 454, Ion Torrent, MinIon, Illumina sequencing, or phi29 genome amplification followed by Illumina sequencing were unsuccessful primarily due to difficulties in library preparation. Nevertheless, successful sequencing was achieved using the Neoprep library system followed by Illumina sequencing, resulting in a closed genome of approximately 142.5 kb. This was consistent with the size of the phage capsid but differed largely from the 242 kb genome size determined by Pulsed Field Gel Electrophoresis.

PCR analysis targeting specific 400 bp fragments dispersed across phi40:2 genome, employing three different sets of primers designed to be specific to the phage and not its host DNA, revealed difficulties in successfully amplifying the phage genome depending on the choice of polymerase and experimental conditions. The Phusion High Fidelity DNA polymerase exhibited the highest success rate, which was further enhanced by optimizing the concentration of $MgCl_2$, as well as the addition of BSA and DMSO. AmpliTaq Gold DNA polymerase did not yield any positive results, while Qiagen Taq DNA polymerase produced weak bands that showed slight improvement under optimized conditions. Invitrogen Platinum Taq DNA polymerase did not yield visible results under standard conditions, but faint bands were observed when using the optimized conditions.

Mass spectrometry analysis of phi40:2 DNA unveiled a unique 346 Da nucleoside modification, likely corresponding to a thymidine modification. This modification differs by only 16 Da from a modification observed in Bacillus phage SP15, known as 5-dihydroxypentyluracyl, which suggests a similar modification with the addition of a hydroxyl group (-OH). A collision- induced dissociation (CID) MS/MS spectra analysis supported the multiple hydroxyl groups in the unknown modification nucleobase. Intriguingly, both phages share a flavin-dependent thymidylate synthase, likely to have diverged into a 5-hydroxymethyluracil (5hmU) synthase, suggesting a conserved mechanism involved in the synthesis of this modified base. We propose that this modification synthesis begins with the insertion of a 5hmU into the DNA during polymerization, followed by further modifications. Alternatively, this protein may have evolved to insert a dihydropentyl group, or similar, from an unknown source. This mechanism introduces the possibility of an unanticipated class of DNA modifications.

Potentially, this modified nucleotide is the reason behind the problems of sequencing or conducting PCR on the phage genome. Given the widespread nature of phages related to other Cellulophaga phages, ranging from the marine environments to the human gut, the geographical distribution and ecological importance of this, as yet, unsequenceable phage is yet to be discovered.

bacteriophages antibiotics evolution

Not Worth the Time: T7 Evolution in the Presence of Antibiotics

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Bacteriophages provide one of the few hopes for stemming the antibiotic resistance crisis. Rather than replacing antibiotics, however, phages are likely to complement them. But will phages be inhibited when used alongside antibiotics, and might it be advantageous to pre-adapt phages for growth in the presence of the drugs? Using the model phage T7 and *E.coli* K12, we investigated whether the phage could adapt to growth in antibiotics and whether the adaptation would be specific to the antibiotic. Phage were separately evolved in sub-MIC concentrations of chloramphenicol (Cm) (2ug/ml), kanamycin (Kn) (8ug/ml), or no drug as control; cells were equilibrated overnight in drug prior to phage addition. Post-adaptation fitness, measured as phage growth rate in the corresponding growth environment, was significantly higher than initial fitness for no-drug control and for T7 grown in Cm; the fitness gain in Kn bordered on significant. However, relative fitness gains were substantially independent of the selective environment: whereas there remained a substantial effect of drug on phage growth rate, that effect had little to do with how the phage was evolved. These limited data suggest that little is to be gained by phage pre-adaptation to growth in the presence of drugs.

Bacteriophages | oxygen stress | facultative anaerobes | P. aeruginosa | phage replication | lytic phages

Low oxygen environments indirectly influence phage replication

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Bacteriophages are ubiquitous and infect host bacteria in all environments including soil, the deep ocean, and the human body. Phages and the facultative anaerobes, like *P. aeruginosa*, that they infect commonly inhabit environments with harsh oxygen stresses. Facultative anaerobic growth and survival depends on their acquisition of free-oxygen and their ability to use alternative molecules like nitrogen as final electron acceptors. *P. aeruginosa* survives in low oxygen environments by sequestering available oxygen in biofilm matrices and through its highly branched respiratory chain containing multiple terminal oxidases and denitrification enzymes. Because phages are highly dependent on their bacterial host cells to survive, changes in host respiration may also influence phage growth. In this study, we use an *in vitro* system with physiologically ‘correct’ tissue oxygen tensions of 5% to accurately model physiological functions of host bacteria infecting human tissues. We determined that a reduction from 21% to 5% oxygen decreased the growth rate of *P. aeruginosa* by 50%, thereby limiting the culture population carrying capacity *in vitro*. Phage infection of *P. aeruginosa* begins with cell surface binding. To explore this stage, we determined the rates at which 90% of phage particles are bound to host cells under the different oxygen levels. The adsorption *k* constants (rate) were 3.05×10^-10 and 5.52×10^-10 under 21% and 5% oxygen, respectively. After binding and virion nucleic acid uptake, the time of virion infection itself (latent period) during which phage proteins are synthesized, nucleic acids copied, and assemble into mature virions can be characterized before “bursting” through cell lysis. We determined the latent periods to be ~26 and ~27 minutes under 21% and 5% oxygen levels. The amount of progeny virions released from infected host cells was about 184 and 179, respectively. We then sought to determine if phage population growth was decreased under low oxygen. PYO2 population growth only reached a density of 7.85×10^9 pfu/ml after 24h at 5% oxygen, whereas it grew to 4.67×10^10 pfu/ml under 21% oxygen. Taken together, low oxygen environments may have influenced *P. aeruginosa* to switch to other forms of respiration, such as using nitrogen instead of oxygen to fuel its metabolic processes. Although phage PYO2 adsorption rate and latent periods were similar under low oxygen, the host cell’s lower energy production appeared to reduce virion progeny production with lower burst size. This small reduction in phage growth over time lead to an 83% lower population density that may be related to decreased host growth under 5% oxygen. Phages are omnipresent with an estimated abundance of 10^31 globally with many of their environments having less than ambient oxygen levels. Although it remains to be determined *in vivo*, lytic phages appear capable of maintaining exponential growth in the body.

Phage-bacteria interactions | machine learning | modelling | Pseudomonas aeruginosa | bacteriophages

Modelling the susceptibility of Pseudomonas aeruginosa to phages at the strain level

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Pseudomonas aeruginosa is an opportunistic pathogen that we use as a model system to study interactions with bacteriophages. From a genomics perspective, the population of *P. aeruginosa* presents a tremendous amount of diversity, with pairs of strains harboring accessory genetic components. These accessory regions include determinants of host-virus interactions such as defense systems, membrane receptors, or prophages that together impact the susceptibility of individual strains to various phages. Our aim is to build models of phage susceptibility that can i) guide the rapid selection of specific virus isolates from phage banks to target given strains; and ii) reveal phage-bacteria interaction determinants through data mining. We performed large activity screens of our phages against hundreds of *P. aeruginosa* isolates. This interaction dataset provides us with thousands of positive and negative interactions while the genomics gives us features to train predictors with machine learning techniques. Our models can be broadly split in white box models which allow introspection and biological interpretation and black box models which enhance efficient phage selection but trade-off interpretation. We show that the population structure of *P. aeruginosa* informs greatly the distribution of phage-bacteria interaction determinants linked to susceptibility. The models we propose yield insights on two fronts: first, we answer an operational question and show how can we rank phages from banks by likelihood of their effectiveness against given strains; and second, we highlight how biological insights can be gathered from data-driven analyses.

Burkholderia pseudomallei melioidosis phage

Exploration of Lysogenic Phages as a Resource for Phage Therapy in *Burkholderia pseudomallei*-Induced Melioidosis

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Burkholderia pseudomallei, a Gram-negative bacterium, is commonly found in tropical soils and freshwaters and is responsible for causing melioidosis in both humans and animals. Melioidosis poses significant challenges for animal treatment due to the emergence of antibiotic resistance, the prolonged and costly nature of effective antibiotic regimens lasting up to 20 weeks, and the complexities associated with decontaminating infected areas. As a result, bacteriophage (phage) therapy is being investigated as a possible treatment for this disease. We study lysogenic phages, the bacterial parasites that incorporate their genome into the host chromosome. We used bioinformatic tools to track the short direct repeat sequences at the 3'end of the recombination event in order to identify prophage regions in bacterial genomes sourced from the GenBank database. This method uncovered over 200 functional prophage regions distributed across 135 completed *B. pseudomallei* genomes. Ten recombination hotspots on *B. pseudomallei* chromosomes were discovered, with P2-like and Lambda-like bacteriophages being frequently observed. Many of these hotspots were found to be associated with tRNA gene sequences, specifically tRNA-phenylalanine, methionine, arginine, cysteine, serine, and selenocysteine. Transcriptomic analysis during normal bacterial growth revealed that the integrase gene play an important role in the phage lysogenic life cycle. Moreover, induction of the prophages resulted in the production of functional phages, thereby establishing a valuable resource for potential phage engineering strategies in future phage therapy applications targeting melioidosis.

phage therapy | bacterial resistance | Escherichia coli | lysis dynamics | physiological characteristics | phage cocktails

PCA-based phage clustering as a powerful tool for effective cocktail preparation

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Background

In recent years, the effectiveness of phage therapy as a treatment strategy for bacterial infections has been reevaluated. Phage cocktails, which consist of a mixture of multiple phages, are used in phage therapy to delay the emergence of resistant bacteria. One approach to preparing highly effective phage cocktails involves using phages that target different receptors. There are generally two main methods for identifying target receptors: (1) creating phage-resistant strains and investigating the mutations between wild-type and resistant strains to study the functions encoded by those genes, and (2) creating a comprehensive library of knockout mutant strains for all (nonessential) gene and examining the ability of phage to infect these strains. Both methods require significant efforts, with the former necessitating advanced knowledge of genomics, such as comparing the whole genome sequences of resistant strains and identifying mutation-induced gene functions, and the latter requiring the preparation of a comprehensive library of mutant strains of the host bacteria that a particular phage infects. Therefore, in this study, we used *Escherichia coli* as a target bacterium and investigated the preparation method of a highly bactericidal phage cocktail based on the physiological characteristics of *E. coli* phage strains, independent of identifying target receptors.

Methods

We isolated phages that lyse *E. coli* derived from fecal samples of mice with colitis using the double-layer agar method. A total of 29 phage isolates were obtained from the sewage of four wastewater treatment plants. For each phage isolate, the lysis dynamics of *E. coli* were measured using optical density (OD) curves. Thirteen phages that exhibited clear lytic properties were selected, and the time at which the OD started to decrease was recorded as the lysis onset time. The lysis duration was calculated by subtracting the lysis onset time from the time when the OD started to increase again, indicating the emergence of resistant bacteria. In addition to these values, the physiological characteristics of phage, including the adsorption rate constant, burst size, titer after one-day cultivation, and lysis spectrum, were investigated. These measured values were normalized and subjected to principal component analysis. Furthermore, the Euclidean distance was calculated using the principal component values of each phage, and hierarchical clustering was performed. A total of 78 phage cocktails were created using pairs of phages belonging to the same or different clusters, and their lytic activity was evaluated based on OD measurements. The change in lysis duration due to cocktail formation, referred to as the extension ratio of lysis duration, was calculated by dividing the lysis duration of the cocktail by the longer lysis duration among the constituent phages. The relationship between the cluster membership of the phages composing the cocktail and the extension ratio of lysis duration was investigated. Additionally, whole genome analysis was performed for each phage, including calculation of Average Nucleotide Identity (ANI) to determine genomic similarity, as well as comparison of tail fibers.

Results and Discussions

When clustering based on physiological characteristics into five clusters and grouping based on genomic similarity (ANI > 95%), the results were consistent. When considering the tail fiber, which provides information similar to the target receptor, we were able to divide the phages into five groups, similar to the physiological clustering and genomic similarity. However, the boundaries between these groups were more ambiguous. When the phages in a cocktail belonged to different clusters, there was a tendency for the extension ratio to be higher. Particularly, among the cocktails with an extension ratio of two or more, indicating a lysis duration more than double, 13 of them were cocktails composed of phages from different clusters, and among those, three cocktails had an extension ratio exceeding six. These results suggest the necessary conditions for designing effective phage cocktails.

Rhizobia | nitrogen fixation | sustainable agriculture | bacteriophage | Sinorhizobium meliloti | phage therapy

Exploring the potential of phage therapy in manipulating the rhizobium-legume symbiosis.

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Rhizobia are involved in fixing atmospheric nitrogen into bioavailable forms in symbiosis with legume crops. They play a crucial role in sustainable agriculture where they alleviate requirements for chemical fertilizers. Many factors affect the growth and viability of rhizobia within the soil and hence impact this important mutualism. One such factor is bacteriophage, obligatory intracellular parasites that can influence the phenotypic as well as genotypic diversity of rhizobia. Infection by bacteriophage can result in cell lysis followed by the release of more bacteriophage into the soil microbiome. Alternatively, rhizobia may bear resistance genes that allow them to resist the bacteriophage infection. In this project, the model organism *Sinorhizobium meliloti*, the natural symbiont of alfalfa plants, is used to study the phage-rhizobium interaction. *S. meliloti* isolates from 33 sites across North Dakotan were isolated and stocked. Selected soil samples were used for the isolation of bacteriophage capable of infecting *S. meliloti*. The isolated phages are being employed to assess the phage resistance-sensitivity profiles across the rhizobium library and will be employed in pan-GWAS analysis to anticipate the phage resistance genes in the rhizobium library. Additionally, series of experiments are being conducted to explore the ability of phage or phage cocktails to eliminate the sensitive rhizobium strains from the soil mesocosms which will pave the path for the potential use of “phage therapy” in manipulating the nodulation.

Prisoner's Dilemma | Pseudomonas aeruginosa | Pf bacteriophages | prophage | temperate phage | filamentous phage | evolution

Prisoner's dilemma: Prophage can enable their bacterial hosts to exploit cooperative bacteria and drive the population to lower fitness

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Evolutionary game theory, specifically the game known as Prisoner's Dilemma, can help explain how cheaters can invade populations despite being less reproductively fit than cooperators. Most strains of *Pseudomonas aeruginosa* contain Pf bacteriophages integrated in their genomes, and these filamentous phages can propagate without lysing their host. Pf phage infection is typically not costly and some benefits to *P. aeruginosa* have been described, including increased antimicrobial resistance. However, cell death may result when multiple Pf phages attack a cell or when Pf replication is uncontrolled. We observed extremely high levels of Pf5 phage replication when *P. aeruginosa* PA14 was experimentally evolved in media simulating nutrients from the cystic fibrosis airway. This Pf5 spread was caused by a mutated repressor gene (*pf5r*) within the prophage, which imposes significant costs on the host bacterium by reducing growth rate and total yield. Despite their lower absolute fitness relative to their ancestor strain, these bacteria containing hyperactive prophage could outcompete the ancestor in direct competition. These *pf5r* mutants are cheaters as releasing phage in the environment selfishly increases their own fitness at the cost of other competitors. It is unsurprising that viruses act selfishly, but more remarkable is that these mutant phage shift competitions between bacterial genotypes to prisoner's dilemma, in which all genotypes evolve lower fitness as the *pf5r* mutation sweeps across the population. This study demonstrates that relationships between bacteria and prophage are unstable and can quickly remodel evolving microbial communities, including potentially during chronic infections caused by *P. aeruginosa*.

Lysogenic phage | microbiome | synthetic biology | engineered phage | Bacteroides

Bacteroides lysogenic phage for payload delivery in the gut

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Changes in the gut microbiota have been implicated in a variety of disease states. In order to fully investigate the role of the microbiome in these diseases, it is critical to systematically probe the role of each individual species of bacteria in a given consortium. Current methods of probing the microbiome are unable to specifically target strains of interest in these diseases. Bacteriophage are a promising avenue to specifically target strain in a native community due to their narrow host range, antibacterial properties, and ability to lyse host microbes. Here, we propose using lysogenic phage, phage integrated into the host genome, to deliver engineered genetic payloads to hosts of interest in an established microbiome. Using *Bacteroides* as a proof-of-concept target, we identified 14 prophage present in *Bacteroides caccae*. We showed that two of these prophage are capable of transducing DNA to a naïve host by using luciferase and antibiotic markers integrated into the prophage genome. As a proof-of-concept payload, we designed an inducible genetic killswitch using an endogenous *Bacteroides* toxin. This system reduced a targeted bacterial population both *in vitro* and *in vivo*. The combination of the prophage with the killswitch could allow for targeted strain removal in an established microbial community in a mouse gut. Such a technique would allow for evaluation of specific bacterial strains on disease.

SPbetaviruses | Bacillus subtilis | environment-dependent interactions

SPbeta-like prophages change *Bacillus subtilis* behaviour under different environmental conditions

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A third of the prophages predicted in *Bacillus subtilis* strains are SPbetaviruses. SPß-like prophages have large genomes, around 130,000 bp and integrate into host functional genes such as *spsM*. Because they feature a reversible active lysogenic lifestyle, meaning they can actively switch between intra and extrachromosomal stages without lysing their host, they affect their host behaviour switching on and off those particular genes. However, little is known about the triggers and the manner they alter their host behaviour and a majority of SPß gene functions are still unknown. To gain insight into prophage-host interactions, we first developed a bioinformatic pipeline to generate a graphical synteny between a group of SPbeta-like prophages, identifying that circa 25% of SPß genes or gene clusters are conserved among the group. Another 25% of SPß genome appear unique to SPß phage, including genes associated with cell lysis and sublancin production. We then used publicly available transcriptomic data to assess what genes are potentially involved in the prophage excision under particular conditions. We noticed that conserved genes globally present low expression variation over the different conditions, although most of them show an increased expression under certain stress conditions. In addition, this analysis allowed us to establish potential correlation between genes or between genes and conditions, based on their expression pattern, indicating their potential role during those processes. Taken together those data show which conditions influence the prophage gene expression profile and whether this behaviour is to be expected in other related prophages, highlighting the relation between prophage genes, their function and their effect on its host.

Phage receptor | Poultry | Phage Biocontrol | Phage Resistance | Salmonella | Foodborne pathogens

Towards Phage-Based Antimicrobial Food Packaging: Developing Electrospun Microfibers Loaded with a Multi-Receptor Phage Cocktail Against *Salmonella*

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Introduction: *Salmonella* enterica subsp. enterica is one of the most prevalent food-borne bacterial pathogens with the highest incidence reported in poultry. The ability of lytic phages to infect and lyse their bacterial target with high specificity makes them ideal candidates for pathogen biocontrol in food production. However, despite their promising features, some hurdles exist to exploiting phage technology for biocontrol purposes to enhance food safety such as the development of phage resistant mutants and having a stable, feasible and effective delivery techniques.

Purpose: The purpose of this study is to design and evaluate a multi-receptor phage cocktail encapsulated in electrospun polyethylene oxide (PEO) microfibers as a potential solution to combat the high variability and emergence of phage resistance in *Salmonella*. The study aims to assess the antimicrobial effectiveness of the phage cocktail *in vitro* and in chicken meat, specifically targeting *Salmonella* Enteritidis.

Methods: Five phages were selected for cocktail composition. This cocktail targets four different receptors: O-antigen, BtuB, OmpC, and rough *Salmonella* strains. *Salmonella* Enteritidis was used to study growth inhibition at different environmental conditions and to investigate the development of phage resistant mutants. Furthermore, the phage cocktail biocontrol efficiency was evaluated on chicken skin. Chicken skin pieces inoculated with 4 $\log_{10}$ CFU/cm^2 were dipped into cocktail suspension containing either 5 or 7 $\log_{10}$ PFU/mL and incubated at three temperatures (25, 15, and 4°C) for 48 hours. To study the antimicrobial effect of the encapsulated phage cocktail, polyethylene oxide (PEO) phage-loaded microfibers were produced using electrospinning. Phage cocktail was electrospun in 12% PEO at 30.3 kV a Elmarco:Nanospider™. Finally, the effect of the phage-loaded electrospun fibers was evaluated in chicken meat. Chicken breast pieces were inoculated to a final concentration of 3 $\log_{10}$ CFU/cm^2 and wrapped in the phage loaded fibers. Wrapped meat was stored at three temperatures (25, 15, and 4°C) and the bacterial reduction was evaluated for up to 48 hours.

Results: Bacteria challenge experiments using *S.* Enteritidis treated with different phage concentrations (MOIs 10^{-1}-10^{3}) showed complete growth inhibition at 25°C and 15°C for 48 and 96 hours, respectively. No cross-resistance to all phages in the cocktail was observed. Biocontrol experiments showed a 3.5 $\log_{10}$ CFU/cm^2 reduction after 48 hours with treatments of 7 $\log_{10}$ PFU/mL at 25 and 15°C, and 2.5 $\log_{10}$ CFU/cm^2 at 4°C. An average of 1 $\log_{10}$ PFU/mg reduction post-electrospinning was observed for all phages in the cocktail. Overall, fibers containing 6 $\log_{10}$ PFU/mg were obtained with the electrospinning method. The PEO phage-loaded fibers showed an immediate release of viral particles upon contact with an aqueous solution. The fibers showed to be stable for up to four months at 4°C. The phage fibers showed an antimicrobial effect against *S.* Enteritidis in chicken meat with 2 $\log_{10}$ CFU/cm^2 reduction observed at 25 and 15°C when compared to the control.

Significance: The bacteriophage cocktail developed in this study holds great promise as a biocontrol tool against *Salmonella*, making it a valuable addition to poultry processing practices for enhanced food safety. Furthermore, this study highlights the potential of phage-loaded microfibers as an innovative packaging material for controlling the growth of *Salmonella* in packed poultry products, presenting a novel approach to ensure product safety.

Temperate phage | phage life cycle regulation | bacterial SOS response | ecology and evolution

Temperate phage encode regulators of the bacterial SOS response to control phage induction

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Bacteriophages (phages) have a tremendous capacity for shaping the ecology and evolution of bacterial communities. Temperate phages, in particular, have the potential to rewire the biology of their bacterial hosts during lysogenic replication when they take the form of an integrated or episomal prophage. In contrast, temperate phages can act as time-bombs upon induction of lytic replication, leading to the depletion of certain bacterial lineages. There are many unanswered questions about the factors that regulate temperate phage life cycles and as a result, how they balance their beneficial and antagonistic relationships with their hosts. In part, this is because many phage genomes and the functions they encode remain uncharacterized (creating so-called “viral dark matter”). To bridge this knowledge gap, I am probing for molecular modulators of phage life cycles using the P2 temperate phage as a model system. P2 is a DNA-damage inducible phage that encodes a small peptide (which we have named ‘PhiR’) that has homology to a bacterial regulator of RecA and the DNA damage SOS response. In this study, I assess how PhiR may function to buffer the bacterial SOS response through RecA interactions that ultimately time prophage induction. I have also found evidence that PhiR may be involved in regulating RecA-dependent adaptive mutagenesis. Lastly, I uncover how widely distributed PhiR-like homologs are across different bacterial lineages and their potential to enhance bacterial fitness. Ultimately, this work highlights how temperate phage tune into the bacterial SOS response to regulate their life cycles and influence bacterial ecology and evolution.

Bacteriophage | wastewater treatment | biofilms | phage therapy

Utilisation of bacteriophage-based biofilm community editing techniques for the enhancement of wastewater treatment efficiency

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Wastewater treatment plants (WWTPs) face increasingly stringent nutrient discharge standards to protect environmental water bodies and human health. Poly-phosphate accumulating organisms (PAOs) are microbes that mediate enhanced biological phosphorus removal (EBPR). PAO are however often outcompeted by glycogen accumulating organisms (GAOs), limiting total phosphorus removal. Previous research has identified a link between GAO abundance and elevated wastewater temperature. Current control mechanisms for limiting GAO require physical or chemical process alterations. However, these alterations have been shown to limit the performance of other aspects of biological nutrient removal (BNR) and enhance nitrogen-based greenhouse gas (GHG) emissions. With the advent of rising global temperatures more regions globally are expected to operate with elevated levels of GAO competition. In addition, some WWTPs already operate at elevated temperatures due to geographical location, seasonal temperature variations and/or heat-treated trade wastewater influent.

We aim to develop a novel bacteriophage-based approach that triggers lysis of GAO as a biological control mechanism for limiting competition between these organism groups. Additionally, with the advent of newly developed targeted genome editing tools, particularly DNA-editing all-in-one RNA-guided CRISPR-CAS transposase (DART), we are exploring new approaches for biological control and enhancement to improve EBPR efficiency. Collectively, this research will demonstrate how biological tools and control mechanisms can be a feasible approach for biofilm community editing and enhancement of WWTP performance.

E. coli ST131 Clade C | multidrug-resistant | bacteriophages | broad host range | phage sensitivity

Assessment and Characterization of Lytic Phages Isolated from Hospital Wastewater against *Escherichia coli* ST131 Clade C Clones

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Escherichia coli sequence type 131 (*E. coli* ST131) is an important pathogen of nosocomial and communal urinary tract infections and bacteremia. Rapid dissemination of multidrug-resistant (MDR) *E. coli* ST131 clade C is becoming a global burden with limited alternative treatment options. Genetically diverse bacteriophages (phages), the virus that kills bacteria, isolated from wastewater have demonstrated lytic activity against various pathogens, including *E. coli* ST131. Additionally, phage treatment has shown a promising outcome in patients infected with MDR pathogens over recent years. In our study, we isolated lytic phages (n = 22) from wastewater (n = 20) collected from four hospitals in Calgary, Alberta. We also assessed intergenomic similarity/difference of isolated lytic phages using VIRIDIC (Virus Intergenomic Distance Calculator). Of the 22 phages, 11 genetically different phages were used for host range screening to determine their lytic activity against clade C, consisting of subclades C1 (n = 8), associated with fluoroquinolone-resistance, C1-M27 (n =9), harbouring extended-spectrum β-lactamase (ESBL), and C2 (n = 8), associated with ESBL CTX-M-14. The analysis showed that 16% (n = 3) to 60% (n = 15) of clade C strains were susceptible to each phage, with two subclade C2 strains resistant to all phages. Furthermore, our preliminary data on genome-wide association studies (GWAS) via Scoary revealed that surface proteins, such as outer membrane usher protein, lipoprotein, pilin, and fimbrial-associated proteins may be pertinent to phage susceptibility. Overall, genetically different lytic phages isolated from hospital wastewater have a broad host range against *E. coli* ST131 clade C clones, and various host surface proteins may serve as receptor for these phages.

Bacteriophage T4 E. coli RNA-binding protein Hfq phage-host interactions

Understanding bacteriophage T4 GoF protein, a putative RNA chaperone

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Upon infection of *E. coli*, bacteriophage T4 relies on host RNA polymerase (RNAP) to propagate the phage's genetic material over that of the host. As phage genetic material is produced, infection viability is sustained through the protection of those phage products. Past investigations have shown that wild-type (WT) T4 does not grow on the host strain Rho026. This strain has a mutation within *rho*, which encodes the main host factor for transcription termination. The inability of WT T4 to grow on Rho026 arises from a decrease in the level of certain middle transcripts, including that of gene 41, the DNA helicase required for T4 DNA replication; a substitution within the T4 early gene *goF* restores growth and increases the level of gene 41 mRNA. This work initially suggested that *goF* might encode a transcription anti-termination factor and that the mutant *goF* is needed to allow growth on the 'super' *rho* mutant strain Rho026. However, other studies revealed that *rho026* encodes a protein that is actually a poorer terminator because of a diminished ability to bind RNA. Consequently, the function of GoF during T4 infection and how the *goF* suppressor mutant promotes growth in Rho026 has still not been elucidated. Using deep blast analyses and the structure predictor program Alphafold2, we have found that GoF belongs to a group of proteins conserved throughout the *Myoviridae* family. Within T4 there are three orthologs, GoF, MotB.1 and Frd.2. Interestingly, all 3 have predicted SM-fold domains at their N-termini. SM-fold domains have been observed in proteins that bind to RNA. Hfq, the major host RNA chaperone, is one such protein in *E. coli*. We postulate that GoF (and MotB.1 and Frd.2) may function as T4 RNA chaperones that work as a phage defense against host RNases. We are currently conducting experiments to test this hypothesis. We hope to leverage this mechanistic knowledge to further understand phage-host interactions.

Erwinia amylovora | Bacteriophage Insensitive Mutant | Fire blight

Phenotypic, Genomic and Transcriptomic Characterization of Erwinia amylovora Bacteriophage Insensitive Mutant

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Introduction: Fire blight, caused by *Erwinia amylovora*, is a prominent concern for the apple and pear farmers due to its drastic economic effects. Traditionally, streptomycin is used for fire blight management, however, health and environmental concerns urged the need for alternative control methods. Lytic phages are considered as promising alternative to antibiotics as pesticide. However, phage resistance development might hinder its wide application as a natural biocontrol agent. Prevention of phage resistance development in the plant pathogenic bacteria requires investigating first the phage resistance mechanism(s).

Purpose: Generating and characterizing *E. amylovora* bacteriophage-insensitive mutant (BIM) resistant to ΦEa46-1 phage to understand *E. amylovora* resistance mechanism against phages and its impact on virulence fitness.

Method: Introducing a novel modification to liquid subculture method to generate BIM by addition of fresh medium and phage on collected cells every week for 3-4s. Analysis of the generated BIM is carried out on phenotypic, genomic, and transcriptomic levels.

Results: The modification of the liquid subculture method able to successfully produce a stable BIM to ΦEa46-1 phage. Difference in the biofilm formation was found between the BIM and the parent strain. Furthermore, significant differences found with Biolog Phenotype MicroArray PM7 plate for Peptide and Nitrogen sources metabolism. Genomic analysis results showed mutations in the two-component Rcs phosphorelay (mutations in rcsB, rcsC, and rcsD genes) and Ion-translocating oxidoreductase complex (rsxC and rsxD genes) when compared to the phage-sensitive parent strain. Mutations lead to DL amino acids insertion, D to V inversion, and promotor regions change. Full analysis of the transcriptomic and phenotypic data still in progress, as well as virulence fitness experiment.

Significance: Two-component Rcs phosphorelay and/or Ion-translocating oxidoreductase complex are playing vital role in phage resistance of *E. amylovora.* Deeper data analysis is required to explore this role in the resistance mechanism and virulence fitness.

Salmonella | bacteriophage; transmission electron microscope; biofilm; phage therapy

Application of a novel polyvalent lytic phage STWB21 as a food preservative and for therapeutic purposes to control *Salmonella typhi*

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Enteric bacteria *Salmonella* is the causative agent for gastro enteropathy and enteric (typhoid) fever. *Salmonella enterica* is a gram-negative, flagellated, rod-shaped bacterium that belongs to the Enterobacteriaceae family. More than 2600 distinct serovars belong to this varied type of bacteria, which are divided into two main categories: (a) Typhoidal *Salmonella* and (b) Non-typhoidal *Salmonella*. *Salmonella* can infect a wide range of hosts from humans to a variety of warm-blooded animals. *Salmonella* may infect a broad range of hosts, including humans and several warm-blooded animals. It is a common foodborne pathogen that is primarily present in poultry, eggs, and dairy products and it may be transferred by human feces, contaminated food, water, and person-to-person contact. Antibiotics have been used more often in recent years to treat bacterial illnesses, which has led to the rise of germs that are multi-drug resistant and it requires to be addressed with an alternative treatment strategy such as phage therapy. Bacteriophages are viruses that can kill their host bacteria without affecting other microflora and co-survive and evolve with their host bacteria. Therefore, bacteriophages have been proposed as an alternative biocontrol agent for bacterial pathogens. *Salmonella enterica* is a foodborne pathogen and causative agent for gastroenteritis, diarrhea, and enteric fever in both humans and animals. The genus *Salmonella* has several serovars, and many of them are recently reported to be resistant to multiple drugs. Therefore, this study aimed to isolate and identify a specific enteric bacteriophage followed by its characterization as a potential biocontrol agent for enteric bacteria.

In this study, a *Salmonella* phage STWB21 was isolated from a lake water sample from Kolkata, India, and found to be a novel lytic phage with promising potential against the host bacteria *Salmonella typhi*. However, some polyvalence was observed in their broad host range. In addition to *S. typhi*, the phage STWB21 was able to infect *S. paratyphi*, *S. typhimurium*, *S. enteritidis*, and a few other bacterial species such as *Sh. flexneri 2a*, *Sh. flexneri 3a*, and *ETEC*. Phage morphology was visualized with FEI Tecnai 12 BioTwin Transmission Electron Microscope (TEM) operating at 100 kV. TEM study revealed that phage STWB21 belongs to the *Siphoviridae* family with an icosahedral head (65 ± 3 nm in diameter) and a long flexible, non-contractile tail (113 ± 6 nm in length). The latent period and burst size of phage STWB21 was 25 min and 161 pfu/cell. As a biological control agent, phage STWB21 exhibited a high temperature (4°C-50°C) and pH (4-11) tolerance for different typhoidal and non-typhoidal *Salmonella* strains. Since *Salmonella* is a foodborne pathogen, the phage STWB21 was applied to treat a 24 h biofilm formed in onion and milk under laboratory conditions to analyze its antibiofilm property, the phage STWB21 was applied to treat a 24h biofilm formed under controlled laboratory conditions. In both cases, a significant reduction was observed in the bacterial population of *S. typhi* biofilm. These findings highlighted phage STWB21 as an anti-biofilm agent for *Salmonella* spp. and showed its application in food industries. It contained a dsDNA of 112,834 bp in length, and the GC content was 40.37%. The genomic analysis confirmed the presence of lytic genes and the absence of any lysogeny or toxin genes. Further, we investigated the effectiveness of phage STWB21 in preventing *Salmonella typhi* from invading mouse liver and spleen tissue and in providing a therapeutic advantage for salmonellosis in a mammalian host. After introducing phage treatment, the phage showed reduced colonization in both the treatment and preventive groups. The results of the study revealed that mice treated with phage both before and after *Salmonella* infection had less tissue inflammation than mice that were not treated, as determined by light microscopy and transmission electron microscopy images. Furthermore, the results suggested that a single STWB21 phage could be efficient in treating *Salmonella* infection without detectable levels of resistance. Our findings collectively showed that the *Salmonella* phage STWB21 has considerable potential for further investigation as a therapeutic and preventative option against *Salmonella typhi*. Overall, the study showed that the novel polyvalent phage STWB21 has a promising ability as a biocontrol agent of *Salmonella spp*. and proposes its application in food industries and for therapeutic purposes.

mycobacteria bacteriophage endolysin lytic cycle

An intramolecular crosstalk in D29 mycobacteriophage endolysin governs the lytic cycle and phage-host population dynamics

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The alarming rise in multidrug-resistant (MDR) and extremely drug-resistant (XDR) tuberculosis has increased the demand for effective alternative approaches to tackling the increasing pathogenic graph. Features such as host specificity, multiplicity at the site of infection and effectiveness against drug resistant bacterial strains renewed interest in phage-based therapeutics. D29 mycobacteriophage, capable of efficiently infecting and killing *Mycobacterium tuberculosis*, projects itself as a potential therapeutic candidate against tuberculosis. The ability of D29 phage to lyse mycobacterial cells is endowed by specific phage-encoded lysin proteins. To overcome mycobacterial cell envelope barriers, D29 phage encodes two functionally distinct enzymes, namely lysin A (LysA) and lysin B (LysB), which ensures the breakdown of peptidoglycan and the outer mycolylarabinogalactan layer, respectively. The ability to break down the mycobacterial peptidoglycan layer projects LysA as a potential therapeutic candidate. However, the regulatory mechanism of LysA during the phage lytic cycle still remains ill-defined. Molecular dissection of LysA suggested it to be a multidomain molecule consisting of two catalytic domains, namely an N-terminal domain (NTD) and lysozyme-like domain (LD), and a C-terminal cell wall binding domain (CTD). Here we show that during D29 lytic cycle, structural and functional regulation of LysA not only orchestrates host cell lysis but is also critical for maintaining phage-host population dynamics by governing various phases of the lytic cycle. We report that LysA exists in two conformations, of which only one is active, and the protein undergoes a CTD-mediated host peptidoglycan-dependent conformational switch to become active for carrying out endogenous host cell lysis. Interestingly, D29 maintains a pool of inactive LysA, allowing complete assembly of phage progeny, thus helping avoid premature host lysis. Additionally, and importantly, we show that the switch reverses post-lysis, thus preventing exogenous targeting of bystanders, which otherwise negatively affects phage propagation in the environment. However, $LysA^{\Delta CTD}$ fails to inhibit exogenous targeting of host cells due to the absence of the structural regulation. We believe that LysA devoid of CTD can be used to target mycobacterial cells for therapeutic purposes. Thus a deep structural and functional insight into the regulatory mechanism of LysA will allow us to develop various efficient phage-based therapeutics to target mycobacterial infection.

phage structure host recognition lambda cryoEM

Lambda phage structure and host recognition

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As the most abundant hunters on Earth, bacteriophages evolved umpteen range of tools to find and bind bacteria, hijack their metabolism to multiply, and lyse the host for progeny release. Studies of every aspect of the phage infection cycle provided us with understanding of fundamentals of living systems and with tools for technological advancement. Relatively slow but persistent spread of antibiotic-resistant bacteria generates demand for diverse antimicrobials. Virosphere seems to be a natural ever-evolving inexhaustible source of such antimicrobials. And yet using bacteria-killing viruses to tackle infections turned out to be not as straightforward as could have been expected.

Little is known about what happens when a phage transits from initial binding of the cell surface to the membrane penetration and genome transfer. A crucial act of decision-making for an individual viral particle remains among the most understudied aspects of viral infection process. Gedankenexperiment approach combined with the fragmented data from a number of experimental systems can help building a convincing model of such a process. Conceivable steps are as follows. Recognition of a specific membrane embedded receptor by a cognate phage receptor-recognition protein, proper orientation of a phage particle on the cell surface, tail opening, trans-membrane and, in many cases, trans-periplasmic channel formation and switch into the genome transfer mode. Nonetheless, the lack of structural data on the subject is considerable, limiting possibilities for quantitative description of infection and phage engineering. One of the reasons for that comes from complexity of host recognition. After all, a phage usually engages several types of specialized appendages - fibers and spikes - into complex interactions with a number of distinct chemical entities on the cell surface before the host recognition reaches irreversible stage and genome transfer begins. Successful host recognition dictates a certain trajectory of binding events none of which can be skipped.

Some phages from the Siphoviridae family provide excellent models for studies of obscured steps of host recognition as they can bypass all the initial and axillary interactions with the cell on the way to the ultimate receptor. If that receptor is sufficiently exposed, the phage can bind it and start infection. Lambda is that type of a phage. It was shown that lambda can infect sensitive Escherichia coli strains though direct binding to the outer membrane protein lamB. One type of tail appendages - central fiber - is necessary and sufficient for lamB recognition and binding. Lambda genome ejection can be triggered with solubilized lamB in vitro in the absence of other cellular components. Lambda ejects its genome into liposomes decorated with lamB recapitulating the chain of events leading to the trans-membrane channel formation in vivo. Studying transition from initial to irreversible host binding at earliest steps of lambda infection benefits from clarity of a radically disentangled system while preserving the possibility to reveal complete mechanics of a key viral decision-making event.

We report a near-atomic resolution structure of bacteriophage lambda tail and its transformation upon receptor binding. The structural analysis suggests limited role of the portal assembly in genome release and evolving of a greater tail-tip autonomy in genome ejection decision making.

phage | antibiotic resistance | adjuvants | antimicrobials | bacteria

Phage Activity Enumeration: Comparison of MD/SP, PLC and Tetrazolium Reduction

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Background. Prevention of rapidly spreading of antibiotic-defeating germs is major challenge today. Using of phages as the adjuvants to antimicrobials to address the threat of antibiotic resistance (AR) is very promising. But developing of simple and sensitive methods for phage activity evaluation in a quick manner is limited, because of phages are “living organisms” with high specificity to bacteria. The optimal management of phage activity evaluation is highly important to prevent infections in the way to improve effectiveness of antibiotic-phage synergistic treatment and slow down the resistance development to both bacteria and phage. Methods. Newly isolated phages of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* from the different natural and industrial sewage environments and with sequenced genome were applied to study comparison of results produced from the methods of MD/SP (Multiple Dilutions on Single Plates), PLC (Phage Liquid Culturing) and BTR (Bacterial Tetrazolium Reduction). OmniLog™ system of redox chemistry to automatically measure cell respiration, a marker for bacterial growth. The phage lytic activity was tested against a large bacterial matrix (103 *P. aeruginosa* and 172 *K. pneumoniae strains*) with the different genetic profiles. Efficiency of plating (EOP) was determined using both the agar plaquing and kinetic clearing methods and the results were correlated in parallel with TR and colony-forming reduction (CFR) in a multi-well-spot format. Results and conclusions. Based on EOP results, six phages of *P. aeruginosa* (Atpa004, Atpa005 and Atpa014) and *K. pneumoniae* (Atkp009, Atkp014 and Atkp016) revealed coverage of about 64-87% for host range. EOP of Most of phages was about two logs less on the test bacterial strains than on the propagating strains. Based on results obtained from Appelmans PLC corelating with plaquing and BR ones, were Identified effective phage/bacteria ratios for each test phage. Phage lytic activity evaluation including pfu/ml and cfu/ml enumerations with correlation-adjustment to TR for a given timepoint, allowed evaluation of phage mutant formation. Further Directions. This approach will facilitate further development of phage lytic activity and emerging phage-resistance mutants’ determination, and selection of candidate therapeutic phages.

E.coli | urinary tract infection | antimicrobial resistance | phylogenic characterization

Phylogenic characterization and antimicrobial resistance pattern of *E.coli* strains

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Urinary tract infection (UTI) is one of the most common nosocomial illnesses, affecting about 250 million people globally each year. The major etiologic agent, *Escherichia coli*, is responsible for up to 90% of cases of UTI. *E. coli* is a Gram-negative, facultative anaerobic rod that is part of the normal intestinal flora. It is exhibiting a very high degree of genetic and phenotypic diversity and classified into 200 serotypes or serogroups based on somatic (*O*), capsular (*K*) and flagellar (*H*) antigens. Only strains of a restricted number of serogroups are pathogenic and are classified into categories or pathotypes based on the production of virulence factors. Uropathogenic *Escherichia coli* (UPEC) are strains that have been isolated from the urinary system. Uropathogenic *E. coli* is the main reason of about 80–90% of UTIs.

UTI affects approximately 150 million people annually and has serious socioeconomic repercussions. Within the framework of the presented research, we studied 90 *E. coli* strains isolated during urinary-genital system infections in March, April and May 2022 in Georgia. To determine the pathogroup, we tested each strain for the presence of uropathogenic *E. coli* (UPEC) specific genes. According to this method, the strain is tested for the presence of three genes, even the confirmation of one gene indicates that *E. coli* is uropathogenic. Of the 90 strains, 81 were identified as UPEC, interestingly, 29 strains had all three genes present, while 22 strains contained only one of the three genes. The *chuA* gene is one of the important markers of UPEC strains. *chuA* is an outer membrane protein with the ability to utilize hemin and is thought to be a key factor in human colonization. As a result of research, we saw that 53 out of 81 *E.coli* strains have this gene. To study the antibiotic sensitivity of the mentioned strains, we used the antibiotics that are currently recommended for UPEC infections. The results show that the strains isolated in Georgia in March-May 2022 are not characterized by broad antibiotic resistance, which is most important to those antibiotics that are prescribed for urological infections, such as nitrofuranthion and fosfomycin, against which only 3-3 of the 90 strains isolated from genitourinary system infections The strain was found to be resistant and none of these strains were resistant to both antibiotics.

Simulated precipitation event | soil viruses | eDNA | nutrient cycles | soil viral ecology | stable isotope probing

Impacts of a Simulated Precipitation Event on Active Soil Viral Communities and Extracellular DNA

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Viruses in soils can potentially impact terrestrial nutrients cycles through auxiliary metabolic genes and by lysing their hosts, releasing nutrients into the soil. The characteristics of the Mediterranean climate found in California (dry summers and wet winters) give us the opportunity to observe how viral and microbial communities are affected by seasonal changes in soil moisture. After a dry season, a precipitation event can cause a jolt of microbial activity, reawakening soil communities, and increasing the number of viral species in grassland soils. Phosphorus concentrations can also affect the number of virus particles produced during an infection, but how phosphorus concentrations affect soil viral communities is largely unknown. Current approaches to study soil viral communities only target specific types of viruses, and the viral signal is heavily occluded by microbial DNA and environmental DNA (eDNA) derived from dead microbial cells and/or extruded from living cells that persists in soils. Here we used stable isotope probing (SIP) combined with viromics to characterize the newly replicated viruses and microbes. This was achieved by creating microcosms with dry grassland soils, simulating a precipitation event by adding natural abundance water or oxygen-heavy water, then adding potassium phosphate to half of the microcosms, and sampling the microcosms weekly for three weeks (T0, 1, 2, and 3 weeks). Using our high-throughput SIP protocol, the DNA was extracted from these microcosms and separated based on density (new viruses incorporated ^{18}O into their DNA, making it denser), and then sequenced. To comprehensively interrogate viral communities in the soil, we also extracted and sequenced the eDNA in the < 0.02 µm size fraction. We benchmarked multiple assemblers and virus detectors to identify viruses and identified 1260 unique viral populations (vOTUs). Given that we did not detect viruses in the eDNA assemblies, we mapped the eDNA reads to vOTUs and determined that less than 0.055% of the eDNA reads mapped to a vOTU. A majority of the mapped viruses (≥ 75% of viral contig length covered) in the eDNA were detected in dry soil but not after the simulated precipitation event, suggesting that viral eDNA accumulates in the soil over the dry season and is quickly utilized upon precipitation. Interrogating soil viral communities from multiple soil DNA fractions (SIP, bulk soil, eDNA) is allowing us to better understand how viruses respond to environmental changes and how virus-host dynamics could impact the carbon cycle and other terrestrial nutrient cycles.

AMR antibiotic ciprofloxacin phage therapy

It takes two to tango: Testing phage-antibiotic pairings to combat AMR pathogens

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Antimicrobial resistance (AMR) is an urgent and expanding threat, with AMR bacteria expected to kill as many as 10 million people annually by 2050. The most common culprit, AMR *E. coli,* was associated with over 600,000 deaths globally in 2019. *E. coli* is also the main cause of urinary tract infections (UTIs), which affect 1 in 2 women and killed 230,000 people globally in 2019. Many antibiotics used for treating UTIs, including the fluroquinolone ciprofloxacin, have seen increasing levels of resistance. New methods to combat AMR are needed.

One promising tool in the fight against AMR is phage therapy – the use of lytic phage to treat bacterial infections. Phage are ubiquitous and diverse, specific for their targets, and cause fewer off-target effects than antibiotics. Most importantly, phage can evolve in response to bacteria and are safe for human consumption. There are currently no FDA-approved phage therapies, but dozens of compassionate use cases have shown favorable results. In the U.S., phage is generally co-administered with antibiotics; this can result in synergistic, additive, or antagonistic interactions. However, there are currently no rules that predict these interactions.

Ciprofloxacin (CPFX) is a fluroquinolone antibiotic that targets bacterial topoisomerases and has previously shown antagonism with phage at some concentrations. *E. coli* and other bacteria can encode a variety of CPFX resistance mechanisms including efflux pumps, target site (DNA gyrase and topoisomerase IV) mutations, porin changes, and proteins that modify CPFX or block its access to topoisomerase. Different resistance mechanisms could alter the intracellular CPFX concentration and affect phage-antibiotic interactions; however, this has not yet been investigated. We hypothesize that bacterial CPFX resistance genes can predict patterns of phage-CPFX interactions.

To test this, we are conducting phage-CPFX interaction screenings on multiple strains of Extraintestinal Pathogenic *E. coli* (ExPEC) at a wide range of phage and antibiotic concentrations, with a focus on uropathogenic *E. coli*. The phage used for screening include ΦHP3 and ΦHP3.1, two genetically similar T4-like phage that encode a topoisomerase. We are also identifying each ExPEC strain's specific CPFX resistance mechanisms through hits to AMR gene databases. This research aims to determine the mechanisms by which bacterial AMR genes impact phage-antibiotic interactions and develop the first rules predicting phage-antibiotic pairings. This could inform rational selection of phage for therapeutic use and help make phage therapy feasible on a larger scale.

Bacteriophages | precision therapeutics | regulatory frameworks | Good Manufacturing Practices | antimicrobial resistance | magistral phage approach

Registered phage products for all– When and How? Let's talk

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Bacteriophages are usually described as precision therapeutics due to their targeted mechanism of action. As a therapeutic, bacteriophages meet the definition of drugs or medicines under most regulatory frameworks. This means product regulation is focused on the assessment of safety, demonstrated efficacy compared to other treatments (*e.g.,* antibiotics), and quality control data. Such frameworks also inherently involve the application of process controlled good manufacturing practices standards, which is seen by many as an expensive undue path for a biological product whose composition can be at times specific to an individual patient. GMP manufacturing is indeed an expensive process, but from a regulator's point of view, quality by design is in place to protect the safety of the public, and high costs is not a justifiable reason to compromise safety.

The same regulatory agencies also have approved pathways (*e.g.*, Expanded Access in the US, Special Access Scheme in Australia, Compassionate Use in Europe) that allow the administration of unapproved medicines to ill patients for whom registered approved medicines are no longer effective. Such pathways are less onerous on development and production methods of the unapproved medicine. In countries like the Republic of Georgia, and other former Soviet Union countries, where bacteriophages have been long approved and used as biological medicines, there is a regulatory process overseen by the Ministry of Health, which hasn't required production under modern GMP standards, but has implemented clear guidelines nonetheless to assure safety and activity of the products, which are widely used.

Due to the antimicrobial resistance crisis, the clinical demand for compassionate treatments has significantly increased around the world, and as a result, the number of clinicians treating patients under this pathway has increased as well. Primarily, their goal is to help patients in need but along the way some are actively trying to document evidence of safety and effectiveness of phage therapy. The challenge for the clinicians has been sourcing well-characterized and purified therapeutic phages that are accepted by the local ethics committees and/or regulatory agencies in a timely manner. In response to this challenge (and many others not yet described here), a self-described pragmatic regulatory framework for tailored phage products, also known as the magistral phage approach, has been established in Belgium. While this arrangement seems to have resolved, at least locally, the issue of product quality controls, the users still report a backlog on the number of patients that can be treated especially due to logistical problems. The data collected suggest the treatments are safe, but no evidence of efficacy has been concretely acquired because nearly every case is a unique phage-pathogen combination.

In parallel, industry has continued the development of fixed-composition bacteriophage products carefully characterized and methodically developed to be active against a particular species of pathogen. These products are typically being used in clinical trials following the traditional medicines regulatory framework. Progress has similarly been slow, and no efficacy data has yet been generated for any indication, *i.e.* there are no approved products yet.

Regardless of the approach taken to help patients in need, everyone agrees randomized controlled trials are required to move the field forward.

While these regulatory challenges may be difficulty to overcome, they are certainly not insurmountable. Phage products for treating plant diseases (*e.g.,* Agriphage™), and controlling animal-borne diseases (*e.g.*, PhageGuard products; ListShield™) have been successfully commercialized and are currently marketed. While these followed different regulatory pathways than human treatment products, similar issues of safety, efficacy, and quality control needed to be demonstrated.

Long-term which of these regulatory approaches might prove more effective in taking phage therapy from experimental treatment to approval by modern regulatory agencies? Should the approaches be combined in some way? We invite the phage community to participate in a discussion with us – either in person or via an on-line survey – about this.

Viromics Nanopore Illumina Human virome

Nanopore and Illumina Sequencing Recover Distinctly Different Viral Populations from Human Gut Samples

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Background: The study of uncultivated viruses through viromics has shaped our understanding of global viral diversity. Long-read sequencing approaches, such as Oxford Nanopore Technologies (ONT) and PacBio, have been widely adopted in bacterial metagenomics, increasing the completeness and quality of metagenome assembled genomes. However, there are few examples of long-read sequencing being used for viromics.

Methods: We sequenced viromes from three human faecal samples using an Illumina HiSeq and ONT MinION, and tested a number of assembler-read combinations including binning approaches. To assess the recovery of viral genomes, we processed the assemblies with geNomad and CheckV. To estimate viral genera, ≥50% complete predicted viruses were processed using vConTACT2 alongside INPHARED.

Results: The ONT assemblies recovered more genomes with ≥50% completeness, more predicted species, and more predicted genera than Illumina, although this varied with assembler used. However, Illumina assemblies recovered more fully resolved genomes than any ONT assembly, and this number was increased by using Phables. Furthermore, ONT assemblies had a higher frequency of genomes that may contain chimeras and/or duplications. To determine the effect of polishing ONT assemblies with Illumina reads, we examined the length of predicted open reading frames (ORFs). Illumina assemblies had a mean ORF length of 142 amino acids, versus ONT assemblies with 127, although this was increased to 133 through polishing with Illumina reads.

Conclusions: The use of hybrid sequencing approaches aids the recovery of viral genomes from natural samples, although ONT assemblies should be treated with caution as they may contain duplications and chimeras. Illumina assemblies still reflect the gold standard for fidelity, and polishing ONT assemblies with Illumina reduces the rate of putative assembly errors. Furthermore, bioinformatic approaches such as binning may aid the recovery of complete viral genomes from Illumina assemblies.

Klebsiella bacteriophage phage Przondovirus sequencing assembly

HYPPA - a hybrid and poly-polish workflow for the complete and accurate assembly of phage genomes: a case study of ten przondoviruses

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Bacteriophages (phages) within the *Przondovirus* genus are T7-like podoviruses belonging to the *Studiervirinae* subfamily, within the *Autographiviridae* family and have a highly conserved genome organisation. The genome size of these phages ranges from 37 kb to 42 kb, encode 50-60 genes and are characterised by the presence of direct terminal repeats (DTRs) flanking the linear chromosome. These DTRs are often deleted during short-read-only and hybrid assemblies. Moreover, long-read-only assemblies are often littered with sequencing and/or assembly errors and require additional curation. Here, we present the isolation and characterisation of ten novel przondoviruses targeting *Klebsiella* spp. We describe HYPPA – a HYbrid and Poly-polish Phage Assembly workflow, which utilises long-read assemblies in combination with short-read sequencing to resolve phage DTRs and correcting errors, negating the need for laborious primer walking and Sanger sequencing validation. Our data demonstrate the importance of careful curation of phage assemblies before publication, and prior to using them for comparative genomics.

hypermodification base modification

Biosynthesis Pathways of Thymidine Hypermodifications

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The DNAs of bacterial viruses are known to contain diverse, chemically complex modifications to thymidine to protect the viral DNA from the endonuclease-based defenses of the bacterial hosts, but their biosynthetic processes are enigmatic. Up to half of thymidines in the Pseudomonas phage M6, the Salmonella phage Vil, and others, contain exotic chemical moieties synthesized via the post-replicative modification of 5-hydroxymethyluridine (5-hmdU) on the genomic DNA. The gene candidates involved in the biosynthesis process were predicted through comparative genomic analysis of the thymidine hypermodified phages. We have determined that these thymidine hypermodifications are derived from free amino acids enzymatically installed on 5-hmdU. Various enzyme classes, such as radical SAM isomerases, PLP-dependent decarboxylases, flavin-dependent lyases, and acetyltransferases, further sculpt these appended amino acids. The combinatorial permutations of thymidine hypermodification genes found in viral metagenomes from geographically widespread sources suggest an untapped reservoir of chemical diversity in DNA hypermodifications.

Antibiotic feed additives | bacteriophage-based feed alternatives | Fusobacterium necrophorum | Streptococcus bovis | in vitro evolution | thermal stability

Development and testing of bacteriophage-based feed additives for cattle

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In the US, beef cattle feedlots commonly use antibiotic feed additives, such as tylosin and virginiamycin, to prevent liver abscesses, which can cause significant economic losses and animal welfare concerns. Ionophores, such as monensin, are also routinely provided to enhance feed efficiency and improve animal performance. However, growing public and regulatory pressure to curb the proliferation of antibiotic resistant bacteria has prompted a need to reduce or eliminate their use. As an alternative, our group is working on bacteriophage-based feed additives that target *Fusobacterium necrophorum*, the primary cause of bovine liver abscesses, and *Streptococcus bovis* complex bacteria with the aim of reducing or eliminating the dependence on antibiotic feed additives.

Using a combination of phage isolation techniques, we identified and characterized fifteen new *F. necrophorum* phages belonging to six genetically distinct groups. These phages exhibited a broad intraspecific host range and could infect various contemporary *F. necrophorum* strains isolated from different regions of the United States. All phages were of temperate origin, with dsDNA genomes ranging from around 36 kbp to 114 kbp. In vitro evolution resulted in the recovery of a potential virulent mutant, φBB37, which inhibited the growth of the high leukotoxin-producing *F. necrophorum* 8L1 strain for over 40 hours in vitro. Phage cocktails proved effective for up to 60 hours in vitro. After minimal optimization, most phages demonstrated high productivity (> 10^9 PFU/mL) and little yield variation during scale-up, highlighting their commercial potential. To assess the safety and in vivo dynamics of *F. necrophorum* phage cocktails, a small-scale animal trial was conducted with nine cattle split into three groups (control, low dose, and high dose). Relative to the control group, *F. necrophorum subsp. necrophorum* was suppressed by 86% over three days (AUC) using high dose (10^{11} PFU), rotated cocktails. *F. necrophorum* concentrations returned to baseline two days after phage feeding was ceased.

Fifteen phages active against *S. bovis* complex bacteria were isolated from rumen fluid and bovine feces. Four were selected for genome sequencing based on their host ranges and killing efficiencies. All four genomes had high similarity, ranged from 33 – 36 kbp, and appear to be obligately lytic. Phages displayed relatively high strain specificity, but were able to maintain host inhibition up to 24 hours in vitro. Appleman's protocol was effective for expanding phage host range from three to seven strains. Since phage-based feed additives are likely to be subject to elevated temperatures during manufacturing or milling, thermal stability was assessed. All phages were initially inactivated by incubation at 50°C for 15 mins or 65°C for 5 mins. Adaptive laboratory evolution significantly increased phage thermal stability, with little inactivation observed after 1 hour at 60°C. Overall, these results support further research into the use of *S. bovis* phages to mitigate ruminal acidosis and improve cattle performance.

phage therapy | citizen science | antibiotic resistance | public outreach | education

Citizen-science outreach and advocacy for advancing phage therapy.

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Bacteriophage research for clinical applications primarily focuses on the biology of phages, their interactions with bacterial hosts, and their impacts on patients. However, there is a lack of research on public perceptions of phage therapy. To address this gap, we have launched a citizen-science outreach project with three main objectives.

Firstly, we aim to educate and raise awareness among the general public about antibiotic resistance and the therapeutic potential of phages. To achieve this, we have developed various accessible resources, including an information leaflet, a card game, a craft activity, and a willingness survey. Together, these resources cater to individuals from different backgrounds and age groups, and some will be translated into different languages to accommodate a diverse audience. The willingness survey will reveal insights into public perceptions and attitudes towards phage therapy, informing our advocacy efforts.

Secondly, we are building a comprehensive biobank of fully characterised phages targeting clinically relevant strains of bacteria. Through a sampling activity, individuals will collect water samples from their local environments. These samples will be sent to our laboratory for phage isolation and characterization, thereby creating a diverse collection of phages from all over the world for phage research. To foster a sense of ownership in their contributions to the project, citizen scientists will be given the opportunity to name their own phage – if isolated from their samples. This publicly accessible collection will support research endeavours in phage therapy for non-commercial purposes.

Lastly, we will collaborate with Public Policy Southampton to engage policymakers on the clinical use of phages in the UK. Our key focus will be on advocating for the removal of Good Manufacturing Practice (GMP) requirements for phage therapy, which would streamline the clinical trials process and facilitate the compassionate use of phages in the UK.

Through this multi-faceted approach, our project aims to bridge the gap between phage research and public perception, while also building a valuable phage biobank and advocating for improved accessibility of phage therapy in the UK. By actively involving the public and policymakers, we hope to create a meaningful knowledge exchange and drive positive change in the field of phage therapy.

Phage-based detection | mycobacterial pathogens

Challenges of developing a phage-based assay to detect low levels of Mycobacteria in blood samples - from cattle to humans via some lions

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Over the last 20 year we have been exploiting a broad host range, lytic phage (D29) as a DNA lysing agent to develop rapid and sensitive tests to detect and identify mycobacterial pathogens. In addition to being very efficient at lysing mycobacteria, phage have another advantage over chemical extraction methods when used as part of DNA-detection tests in that only viable cells are lysed, and therefore successful lysis using phage also reports on the viability of the cell detected. We have now developed a method that can successfully detect mycobacteria within 6 h, which has been commercialised as Actiphage test. During these studies we have discovered new features of the phage-host interaction that has allowed us to better understand and improve our test methods and have shown that the method can be used to diagnose mycobacterial infections in a range of animals, including exotic species such as lions and elephants.

However, for any new biotech product to flourish, there has to be a commercial market, so more recently we have shown that Actiphage can be used to detect TB in human blood. Using phage D29, which, only efficiently infects metabolically active *Mycobacterium tuberculosis* (Mtb), an initial proof of principle study found viable Mtb can be detected in peripheral white blood cells isolated from patients with active pulmonary TB in immunocompetent patients (Sn = 73%; Sp = 100%). Positive phage assay results were associated with a range of other clinical indications of infection, suggesting that low-grade, bacterial dissemination is a feature of poorly controlled disease and occurs more commonly than has been generally reported in this group. In a second, follow up, prospective study focussing on identifying progressive infection in asymptomatic pulmonary TB contacts, we have recently found evidence for a detectable bacteraemia in earlier stages of progressive TB infection.

This talk will review what we have learned about phage host interactions during the development of Actiphage, and outline how we are now adapting this method to ask new questions. Once again phage have been shown to be an invaluable tool to reveal new things about their host cells.

"The real voyage of discovery consists not in seeking new landscapes, but in having new eyes" Marcel Proust, (1871-1922)

Swift et al. (2020) The development and use of Actiphage® to detect viable mycobacteria from bovine tuberculosis and Johne's disease-infected animals. Microb Biotechnol. 13:738– 746 doi: 10.1111/1751-7915.13518

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UPEC | anti-phage defence | yeast recombineering | CRISPRi | small heat shock proteins

Understanding and exploiting phage-host interactions for enhanced phage therapy

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Antimicrobial resistance (AMR) presents a significant global health, economic, and social burden. With traditional antibiotics losing effectiveness against drug-resistant bacterial pathogens, phage therapy, which uses bacteriophages (phages) to treat bacterial infection, offers a promising alternative. The success of phage therapy relies on productive interactions between phages and their hosts. Thus, understanding these complex interactions remains an important goal, as it will enable us to select natural phages more efficiently for a given task, and engineer phages with enhanced capabilities. We present progress towards this goal through the investigation of three different model phage-host systems (*Microviridae, Autographiviridae,* and *Mesyanzhinovviridae*) and two diverse classes of genes (small heat shock proteins and anti-phage defence systems and their effect on phage susceptibility.

First, we determined the role of two small heat shock proteins (sHsps) – IbpA and IbpB – during *Microviridae* φX174 replication. sHsps are a family of molecular chaperones produced by bacterial cells under stress, which prevent the irreversible aggregation of proteins. IbpA and IbpB, a class of holding modulators or "holdases", bind partially folded proteins and await ATP-driven folding chaperones for refolding. These two proteins have recently been shown to be highly upregulated during φX174 infection of *Escherichia coli* C. Here, using a hybrid approach of CRISPR interference (CRISPRi) and genomic knockouts to disrupt the *ibpA/B* genes, we show that surprisingly, IbpA and IbpB appear nonessential for φX174 infection.

Next, we focused on uropathogenic *E. coli* (UPEC) as a model system to holistically investigate the role of host factors on phage susceptibility. We assembled a set of 35 multidrug-resistant (MDR) clinical UPEC strains from 15 sequence types and a panel of eight recently isolated UPEC phages. We experimentally determined the host-range of the phages and assessed their attachment efficiency. We found that most of the non-infection events could be attributed to failed adsorption, with the action of host defence systems likely accounting for the remaining cases. Next, we built a bioinformatics pipeline integrating PADLOC-DB, DefenseFinder, and CRISPRDetect to determine anti-phage defence systems involved in phage susceptibility. We identified 41 putative anti-phage defence systems in our UPEC collection. We found that one strain contained as many as 12 defence systems and was resistant to seven UPEC phages. To extend this work further and determine its generalisability across the UPEC strains, we identified 74 anti-phage defence systems in the genomes of 409 UPEC isolates from the Refseq database. We also compared the prevalence of defence systems in UPEC to Non-UPEC, and we discovered significant differences in several systems, such as CRISPR/Cas.

In parallel, we are further developing methods to clone phage into yeast to enable their manipulation and augmentation with heterologous genetic functions to expand their host-range. Examples of modified phages using yeast recombineering and OrthoRep *in vivo* continuous evolution system will be presented.

In summary, by leveraging the knowledge of phage-host interactions and applying genetic engineering approaches, we will optimise the therapeutic potential of phages, improving the overall efficacy of phage therapy.

Klebsiella pneumoniae | Bacteriophage

Identifying the Receptors of 30 Lytic *Klebsiella pneumoniae* Bacteriophages and Expansion of their Host Range

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Klebsiella pneumoniae is a common nosocomial pathogen, with high incidence of extended-spectrum-β-lactamase-resistance leading to poor patient outcomes. With the continued emergence of antibiotic resistant *K. pneumoniae*, including highly resistant strains producing the blaKPC carbapenemase, alternative treatment options are under investigation. Bacteriophages, as major natural predators of bacteria, are a promising alternative antimicrobial, but their use requires thorough characterization of candiate phages. This work aims to determine the receptors used by 30 lytic bacteriophages, and determine if phages are able to evolve in vitro to overcome loss of their receptor. A collection of 30 virulent phages were isolated against a panel of clinically relevant *K. pneumoniae* isolates carrying a blaKPC allele. Phage-resistant bacterial mutants were isolated against each phage, and the mutant genomes sequenced to identify mutations associated with phage resistance. Mutations in the bacterial capsule, LPS synthesis loci, or outer membrane proteins were associated with phage resistance. Complementation of the mutant genotypes and phage adsorption phenotypes is ongoing.A system to select for phage mutants that are able to overcome bacterial resistance was developed, exploiting the natural ability of phages to evolve along with their predators to see if these phages can expand their host range. In experiments to date, only a subset of phages appear able to overcome host resistance by evolution *in vitro*. This work will assist in identifying phages that would make the most effective therapeutic cocktails while overall reducing the possibility of an ineffective treatment due to the emergence of phage resistance.

Pseudomonas aeruginosa | Candida albicans | antimicrobial peptides | AMPs | combination therapy

Bacteriophage and Antimicrobial Peptide Combination Treatments Against *Pseudomonas aeruginosa* and *Candida albicans*

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Pseudomonas aeruginosa and *Candida albicans* are opportunistic pathogens of great concern due to their broad arsenal of virulence factors and resistance to antimicrobial drugs. These pathogens are among the most common agents in hospital-acquired infections and are often co-isolated from lung and wound infections. Furthermore, mixed-species biofilm formation often plays an important role during these infections, providing additional protection against antimicrobial compounds. *P. aeruginosa* preferentially binds to *C. albicans* hyphae in mixed-species biofilms, shielding *C. albicans* from environmental stresses, including antimicrobial treatment. Bacteriophage (phage) therapy and antimicrobial peptides (AMPs) present as encouraging approaches as treatment against such infections. Phage therapy, however, provides limited remedy for fungal infections. There also are no known lytic viruses that infect *Candida* spp. Therefore, the present study aims to develop novel treatments against *P. aeruginosa* and *Candida* spp. mixed infections, using combinations of phages and antimicrobial peptides. Specifically, we analyzed phage-AMP combinations against single- and dual-species populations growing planktonically and in biofilms. We tested 4 different phages (LUZ19, PEV2, 14-1, YuA) and 7 different AMPs (chCATH-2, histatin-5, Cm-p5, AurH1, IDR-1018, human β-defensin-3, bovine lactoferricin) exhibiting antibacterial, antifungal, and/or anti-biofilm activities. First, we examined phage stability and adsorption kinetics in the presence of AMPs. Several antagonistic combinations were observed, which were excluded from further testing. Next, suitable candidates were assessed against planktonic *P. aeruginosa* and *C. albicans* cultures via lysis profile analysis, as well as their ability to degrade established single- and dual-species biofilms *in vitro*. We identified phage-AMP combinations with increased effectiveness against *P. aeruginosa* and *C. albicans* planktonic cultures and mixed biofilms in comparison to single phage or AMP treatments, indicating additive activity between these two types of antimicrobial agents.

bacteriophage | MDR Pseudomonas aeruginosa | carbapenem antibiotics | antibiotic sensitivity

Combinations of novel bacteriophage are efficacious against MDR *Pseudomonas aeruginosa* and restore sensitivity to carbapenem antibiotics.

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Combinations of novel bacteriophage are efficacious against MDR Pseudomonas aeruginosa and restore sensitivity to carbapenem antibiotics.

Protein folding | molecular modeling | phage viability | stability effects | ΔΔG | phylogenic conservation

Predicting the viability of coat protein mutations in the bacteriophage ϕX174 using molecular modeling

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For proteins to function, they must first fold and then bind appropriate partners in stable ways. Failure to do either of these will usually be lethal. Molecular modeling provides a way to predict the effects of many potential mutations on folding and binding stability—and thereby, a way to predict mutational viability of future viruses. To test this concept, we studied mutations in the coat protein of the Microvirid bacteriophage φX174. We engineered all possible variants at 21 sites of the protein's 176 residues, determined the viability of each, and tested the ability of molecular modeling to predict viability. We found that just over half of the variants are viable (223 of 420, or 53%). We developed a new type of logistic-regression model to assess how well predicted stability effects (ΔΔG) explain phage viability. The model is novel in that it assumes predictors interact multiplicatively (rather than additively) and that the logistic function asymptotes at a value below one. The logistic model indicates viability drops quickly as mutations exceed ΔΔG of +4 kCal/mol. However, we also find that that molecular modeling has only a moderate capacity to predict viability in this phage protein: mutations predicted to be highly destabilizing are indeed inviable, but most mutations are predicted to have small stability effects, and nearly half of these are still inviable. Our results support the view that there are many ways for protein to be non-functional: being unstable is just one of them. Finally, we compare the predictive power of molecular modeling with phylogenic conservation as a different source of information about viability.

Salmonella phage | phage isolation | host range

Characterization of a Diverse Collection of Salmonella Phages Isolated from Tennessee Wastewater

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Salmonella enterica is one of the most prevalent bacterial foodborne pathogens. As with other pathogens, there has been a concerning trend of increasing prevalence of antibiotic resistant isolates, including multi-drug resistant strains from both human outbreaks and in the food production environment. *Salmonella* bacteriophages are currently used in biocontrol applications and have potential for use as therapeutics. For the development of effective bacteriophage applications, diverse collections of well-characterized bacteriophages that are active against a broad range of target bacteria and that contain phages with both narrow and broad host ranges is an essential resource. To explore the diversity of phages present in wastewater effluent in eastern Tennessee and develop a collection of eastern Tennessee *S. enterica* phages for future use, samples were enriched, isolated, and purified from a collection of 24 *Salmonella* host isolates. From these isolates, ten phages were selected for further characterization. Morphology was determined with transmission electron microscopy, host ranges were characterized using an efficiency of plaquing assay, and comparative genomic analysis was performed to determine taxonomy. The characterized phages belong to the three major bacteriophage morphotypes (three myoviruses, two podoviruses, and five siphoviruses) Five different genera (*Ithacavirus*, *Gelderlandvirus*, *Kuttervirus*, *Tlsvirus*, and *Epseptimavirus*) are represented within these phages, with two belonging to established species, and eight belonging to novel species. When evaluated by efficiency of plaquing assay, these ten phages showed activity against 23 out of 24 *Salmonella* serovars, with host ranges varying from a single host to half of all tested serovars. Additionally, two phages displayed polyvalent activity against both *Salmonella* isolates and the lab strain *E.coli* B. The phages described here illustrate the diversity of *S. enterica* phages present in wastewater effluent and provide a collection of characterized phages from eastern Tennessee that may be of use in future phage-based applications targeting *S. enterica*.

bacteriophage | decontamination | *B. anthracis* | food processing | antibiotic therapy

"All's Fair in Love and Lytics": Polyvalent Phage Screening for Decontamination of *B. anthracis*

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Lytic bacteriophage (phage) are natural viral predators of bacteria that target and kill their host(s) following infection, and therefore are of interest for a range of applications including as alternatives for antibiotic therapy, pathogen destruction during food processing, and environmental decontamination in medical and other settings. However, the process to screen phage that specifically target the microbial host of interest can be involved, sometimes requiring screening materials from unconventional sources against dangerous pathogens. Despite the historical definition of a phage as targeting only a single, highly specific bacterial species or even sub-strain, a few cases of "polyvalent" phage that target multiple closely related species, linked at the genus level, have been reported in the literature. The potential value of identifying polyvalent phage is multi-fold, including advancing towards understanding the potential for identifying "broad-spectrum" phage which have lytic activity towards many microbes, allowing for more targeted within-genus screening using lower-threat simulant hosts, and supporting the fundamental understanding of structure-function relationships between phage and their host(s). *Bacillus anthracis* is a biothreat microbe for which there is interest in developing phage-based decontamination strategies, however working with the dangerous pathogen requires a significant training and laboratory infrastructure investment. Therefore, mitigating approaches include developing computational screening methods, using safer simulant organisms, and assessing the applicability of the near-neighbor polyvalent phage approach for this microbe. In this work, we assess a series of phage reported in the literature or through unpublished communication to act against at least one of the below microbes to assess their lytic activity against multiple other *B. anthracis* near-neighbor *simulants*, including *Bacillus subtilis*, *Bacillus thuringiensis*, and *Bacillus cereus*, among others. We seek to understand the structure-function relationship between the phage activity and the genetic and structural composition of the microbe using both computational and benchtop methods. Initial results from the screens will be presented and computational analytics issues will be discussed.

Mycobacteria Bacteriophage Genome

TribleTrouble: A Novel K3 Phage with Therapeutic Potential

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Bacteriophages (phages) are viruses that can infect and destroy bacteria. This phenomenon was first observed in the early 1900s, and researchers immediately recognized phages could potentially be used to control bacterial infections. Today, with the pressing health concern of antibiotic resistance, an alternative approach to battle drug resistant bacteria is crucial. Before the therapeutic potential of phages can be realized, it is vital to discover and characterize phages from the environment. The purpose of this study was to isolate and characterize a novel phage that can infect members of the genus *Mycobacterium*, some of which are notable human pathogens.

Starting from a soil sample collected on Western Kentucky University's campus, a phage capable of infecting *Mycobacterium smegmatis*, a fast-growing nonpathogenic strain, was isolated, grown and purified. Electron microscopy revealed that the phage, named TribleTrouble, is a member of the Siphoviridae because it has the characteristic icosahedral head and non-contractile tail. Restriction analysis of TribleTrouble's purified genomic DNA suggested it has a high G+C content. To determine TribleTrouble's relatedness to other phages, we sequenced its entire genome. This analysis revealed it is a member of the K cluster of mycobacteriophages. Interestingly, K cluster phages can infect both fast and slow growing mycobacteria such as *M. tuberculosis*. TribleTrouble is now part of a national phage collection and is an important new resource for scientists studying phage therapy.

Bacteriophage | Pseudomonas | Canine skin infection | otitis

Bacteriophages to treat canine skin and ear infections: Isolation and characterization of *Pseudomonas aeruginosa* bacteriophage.

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Bacteriophage therapy, which uses lytic virus as antibacterials, is acknowledged as an alternative treatment option to address the global epidemic of antimicrobial resistance in bacteria. Skin infections and otitis externa caused by *Pseudomonas aeruginosa* is very common in canine practice. Treatment challenges are significant for the biofilm producer, inherently drug resistant, and now increasingly fluoroquinolones resistant *P. aeruginosa* infections. In veterinary medicine, success of phage therapy has been shown in livestock animals and in food safety. However, phage therapy is not adequately explored in small animal medicine. This study aims at tackling drug resistant pathogens in canine dermatology. In this study, we have isolated three lytic anti-*Pseudomonas* bacteriophages from the sewage wastewater using clinical isolates of *P. aeruginosa* as the propagating host. The characterization of one virulent phage is presented. The biological activity of the phage generates clear, approximately 1 mm lytic zones. The phage has shown high lytic activity against 20 clinical isolates of *P. aeruginosa* isolated from the pyoderma and otitis cases of canine patients. The in-vitro growth kinetics of *P. aeruginosa* versus phage displayed an exponential clearing of bacteria in liquid cultures, showing lowest OD_{600} at 4.5 h. At present, the sequencing of the bacteriophage genome is underway, which will provide further information on its taxonomic placement and characteristics.

Salmonella | Nontyphoidal salmonellosis | prophage | serovars | virulence

Diverse prophages of *Salmonella enterica* show potential role in disease and diversity

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Nontyphoidal salmonellosis is an important foodborne and zoonotic infection, causing significant global public health concern. Diverse *Salmonella* serovars are multi-drug resistant and encode for several virulence indicators, however, little is known on the role prophages play in driving these phenotypes. Here, we determined the potential contributions that prophages make to the diversity and pathogenicity of *Salmonella*. To do this, we explored 75 genomes from publicly available strains, representing the 15 most prevalent *Salmonella* serovars found in the United Kingdom. All prophage elements from the genomes of the strains were extracted using PHASTER tool and classified based on their completeness. The genomes of the intact prophages were further analysed for the presence of potential virulence factors using VFanalyzer platform within the Virulence Factor Database. Furthermore, we constructed phylogenetic relationships to reflect the genome diversity of the representative intact prophages using Mega 11. We identified 615 prophage elements from the genomes of the examined *Salmonella* strains and revealed that 195 prophages are intact, 332 incomplete and 88 were questionable. The prophages were found to be more prevalent in *S.* Heidelberg, *S.* Inverness and *S.* Newport (encoding 51-58 prophages each) compared to *S.* Agona, *S.* Braenderup, *S.* Bovismorbificans, *S.* Choleraesuis, *S.* Dublin, *S.* Enteritidis, *S.* Infantis, *S.* Java, *S.* Javiana, *S.* Stanley, *S.* Typhimurium and *S.* Virchow (with 29-53 prophages each). Cumulatively, 2760 virulence factors were detected in the genomes of the intact prophages and associated with cellular functionality being linked to effector delivery/secretion system (73%), adherence (22%), magnesium uptake (2.7%), resistance to antimicrobial peptides (0.94%), stress/survival (0.4%), exotoxins (0.32%) and antivirulence (0.18%). Various close and distant clusters were formed among the prophages suggesting different lineages and associations with prophages of other *Enterobacteriaceae*. Our data showed strong correlation between *Salmonella* prophages and numerous virulence factors, and contributing to diversity, pathogenicity and success of specific serovars. Further work would focus on how these prophages drive the evolution and selection of prevalent serovars responsible for salmonellosis in the United Kingdom.

viral identification | metagenomics

Caution is needed when combining viral identification methods: Insights from benchmarking *in silico* approaches for viral discovery

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Viral analyses require accurately differentiating viral from cellular sequences in complex metagenomes. As viruses have no universal marker genes, high mutation rates, and reference databases that do not well-represent their diversity, this remains challenging. An increasing number of bioinformatics tools have been developed to recover viral sequences from metagenomic datasets. Each of these tools has its own biases, making it challenging for researchers to know which tool(s) would be best suited to their specific application. Many researchers combine the output of multiple viral identification tools ("tools") in an attempt to exploit the unique strengths of each to distill a set of higher confidence viral sequences. However, this approach has yet to be rigorously benchmarked.

Hypothesizing that a multi-tool approach would discover more viruses without greatly increasing contamination, we evaluated 27 published viral identification tools. We benchmarked 63 combinations of 6 tools ("rulesets") that met our preliminary requirements using a mock environmental metagenomic dataset composed of publicly available viral, bacterial, archaeal, fungal, plasmid, and protist sequences. In addition to four single-tool rules (based on VirSorter, VirSorter2, DeepVirFinder, and VIBRANT), we also developed two multi-tool "tuning" rules. These rules use Kaiju, CheckV, and VirSorter2 to refine our predictions, adding sequences with particularly viral features ("tuning addition") or removing sequences with particularly cellular features ("tuning removal"). We then applied these rulesets to different aquatic metagenomes (fresh and saltwater, drinking water, wastewater) to evaluate the impact of habitat on performance.

We found that 6 rule rulesets had MCCs that were statistically equivalent ($p_{adj} \geq 0.05$) to the ruleset with the highest MCC (MCC=0.75). These "high MCC" rulesets all included VirSorter2, 5 of the 6 included our "tuning removal" rule, and none used more than 4 of our 6 rules. DeepVirFinder, VIBRANT, and VirSorter were each found once in the "high MCC" rulesets, but never in combination with each other. We further determined that 2 to 5 rule rulesets increased precision compared to the single-rule ones ($p_{adj} \leq 0.05$); and that 4 and 5 rule rulesets increased recall compared to 1 to 3 rule ones ($p_{adj} \leq 0.05$). From the environmental metagenomes, we found that different rulesets were better suited for virus vs cellular-enriched metagenomes. In this talk, I will share our ruleset recommendations for different environments and research questions, and provide a blueprint for intentional, data-driven validation of viral identification tool combinations.

Overall, we found that caution is necessary when combining viral identification tools. While combining tools does increase viral recall, it comes at the expense of more false positives. Ultimately, by increasing the number and quality of viruses identified from metagenomes through intentional, data-driven combination of tools, this work will improve ecological insights with far-reaching implications for human and environmental health.

Bacteriophage therapy | antibiotic resistance | temperate phages | phage-antibiotic synergy | Pseudomonas aeruginosa | bacterial eradication

Temperate phages can re-sensitize *Pseudomonas aeruginosa* to antibiotics

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With a decline in antibiotic effectiveness, there is a renewed interest in bacteriophage (phage) therapy. Phages are bacterial-specific viruses that can be used alone or in combination with antibiotics to reduce bacterial load. Most phages are unsuitable for therapy because they are 'temperate' and can integrate into the host genome, protecting the host from subsequent phage infections. However, the integrated phage can be awakened by stressors such as DNA-damaging antibiotics. Parallel work from the lab uncovered strong synergy between a model temperate phage and sublethal ciprofloxacin, a fluoroquinolone, resulting in eradication of the phage's *Escherichia coli* host. This project explores the potential of combined temperate phage-antibiotic synergy against a clinically relevant multi-drug resistant pathogen, *Pseudomonas aeruginosa*. Temperate phages infecting *P. aeruginosa* PA14 were isolated from the McMaster Wright clinical isolate collection. These phages were screened for synergy with six antibiotics (ciprofloxacin, levofloxacin, meropenem, piperacillin, tobramycin, polymyxin B), spanning four classes, using checkerboard assays. Surprisingly, our screen identified phages that can synergize with each of these antibiotics, despite their widely differing mechanism of action. One of the strongest pairings with ciprofloxacin was studied in further detail to understand the mechanism. This combination reduced the survivor count 10^8 fold compared to the untreated host, corresponding to complete bacterial eradication. This was a result of the antibiotic preventing phage dormancy. Ciprofloxacin also worked in combination with multiple phages even in clinical hosts resistant to the antibiotic, re-sensitizing the bacteria. Preliminary results show that combination of phage and piperacillin, a common anti-pseudomonal antibiotic, can increase *Caenorhabditis elegans* lifespan after an established *P. aeruginosa* infection. This is the first *in vivo* testing of temperate phage-antibiotic use. Overall, our findings indicate that temperate phages can act as adjuvants alongside our currently available antibiotics, drastically expanding their therapeutic potential.

Mycobacterium smegmatis | bacteriophage | MooMoo | gene toxicity | recombinant plasmids

Identification of toxic gene products from *Mycobacterium smegmatis* phage MooMoo

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Bacteriophage MooMoo is a temperate phage that was enriched from a water sample using Mycobacterium smegmatis as the host. Based upon published observations, we hypothesized that MooMoo may encode genes that are toxic to Mycobacterium smegmatis, when overproduced. Five genes (*gp87*, *g88*, *gp89*, *gp90*, and *gp91*) were tested for toxicity because they encode small proteins that have no known function. Each gene was amplified from the phage genome by polymerase chain reaction (PCR) and cloned into an Escherichia coli/M. smegmatis shuttle/expression vector. The recombinant plasmids were propagated in E. coli, verified by PCR, then moved into M. smegmatis cells. The cloned genes were induced and cell growth was monitored on plates. The toxicity of each gene was scored on a Toxicity Index (TI) from 0–5, with 0 corresponding to no toxicity (abundant growth) and 5 representing the strongest toxicity (no growth). MooMoo *gp87* displayed the strongest toxicity and was chosen for further analysis. To determine how *gp87* interferes with M. smegmatis growth, we performed a protein-protein interaction assay using a bacterial two-hybrid screen. This analysis identified an interaction between *gp87* and the host metabolic machinery. Future experiments include modeling the interacting surfaces of the proteins and attempting to isolate host mutants that suppress the toxicity. Our analysis represents a general approach for elucidating gene function and may identify potential new targets for therapeutics.

Fusobacterium necrophorum | phages | bovine | liver abscess | antibiotics

Fusobacterium necrophorum phages as a potential tool for bovine liver abscess management

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In the beef cattle industry, prophylactic treatment with antibiotics targeting putative etiologic agent of liver abscess formation, Fusobacterium necrophorum, has been shown to be inadequate, resulting in notable product losses ($60 million per year)—a situation that would considerably worsen if antibiotics were curtailed. Concomitantly, public sentiment and stringent regulations are increasingly rallying against antibiotic feed additives, given their perceived role in fueling the proliferation of resistant bacterial strains. To address this need, our work has looked to phage therapy as a potential alternative, or adjunct, to antibiotics. Here, we present the isolation, characterization, and subsequent genetic profiling of six novel phages with the ability to infect *F. necrophorum*. Our comprehensive assessment of their capacity to inhibit the growth of the *F. necrophorum* subspecies necrophorum 8L1 (FN8L1) has yielded interesting results, both singularly and in combined scenarios. Notably, while all phages possessed protein-coding sequences akin to those linked with lysogeny, two among the six did not form lysogens. Moreover, no bacterial immunity mechanisms could be detected following exposure to one of our phage isolates, ϕBB. Upon testing the host ranges of these phages, it emerged that four of the six phages were confined to a single Fusobacterium subspecies. However, both ϕKSUM and ϕBB proved capable of infecting multiple subspecies. Beyond this, we documented the efficacious application of four of the isolated and characterized phages as part of high-dose, sequential treatment protocol, utilizing two distinct rotating cocktails to suppress challenge strain FN8L1 growth in the rumen of nine cannulated calves. These findings underscore the potential of certain lysogenic phages as therapeutic agents for sustained bacterial population control, particularly when lytic phages prove challenging to acquire.

Flow Cytometry | Viral Tagging | Phages

Capturing phage-host pairs with viral tag and grow

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Bacteriophages (phages) are viruses that infect bacteria and are one of the most abundant biological entities on the planet. Continued advances in culture-independent sequenced-based analyses have allowed viral discovery to advance at an astonishing rate, revealing hundreds of thousands of new uncultivated viral genomes to be further explored. The problem with these approaches, however, is that there is currently no way to accurately associate a bacteriophage with its host and answer the fundamental question: who infects whom? Here we develop a scalable approach to link phages to their host cells using the fluorescent activated cell sorting protocol, Viral Tag and Grow, which utilizes a growth assay allowing for characterization of phage-host pairs through successful lytic infections. In this study, the host *Pseudomonas aeruginosa* PAO1M was infected with *Pseudomonas* phages phiKZ and PEV2. We observed a fluorescent shift for our nucleic acid stained phages when they were infecting the unstained host compared to the normal host's cytogram, indicating adsorption of the phage to the host. Observed shifts in fluorescence were directly correlated with changes in the phage adsorption within the sample. The infection couldn't be characterized through a growth assay due to inaccurate sorting caused by instability within the sample droplet during sorting. Currently, the data supports previous findings that adsorption can be detected using flow cytometry, while characterization of the infection through a sorted growth assay requires further development. Further investigation of the ability to sort single phage-host pairs and characterize infection through a growth assay may illustrate the ability for this method to become a scalable approach for phage-host discovery of obligately lytic phages and answer the fundamental question: who infects whom? These findings provide the foundation for a scalable approach to phage-host discovery that can be used for phage therapy library construction and exploring ecological population structure.

bacteriophages | antibacterial agents | therapeutic phages | pharmacodynamics | extracellular search | phage adsorption rate constant | bacterial size | virion diffusion rates

Why R?

Stephen T. Abedon [1*]

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Bacteriophages (phages) are viruses of bacteria and have been used as antibacterial agents now for over one-hundred years. The primary pharmacodynamics of therapeutic phages can be summed up as follows: phages at a certain concentration can reach bacteria at a certain rate, attach to bacteria that display appropriate receptors on their surfaces, infect, and (ideally) kill those now adsorbed bacteria. Here I consider the rate at which phages reach bacteria, during what can be dubbed as an 'extracellular search'. This search is diffusion driven and can be described by what is known as the phage adsorption rate constant. That constant in turn is thought to be derivable from knowledge of bacterial size, virion diffusion rates, and the likelihood of phage adsorption given this diffusion-driven encounter with a bacterium. Here I consider only the role of bacterial size in encounter rates. In 1932, Schlesinger hypothesized that bacterial size can be described as a function of cell radius (R, or R1), as based on non-phage-based theorizing of Smoluchowski (1917). The surface area of a cell—what is actually encountered—varies however instead as a function R^2. Here I both provide and review evidence indicating that Schlesinger's assertion seems to have been correct.

This supposedly is about to be published in the new MDPI journal, *drugs and drug candidates* (lower case intentional).

Also, see http://encounter.phage.org

Burkholderia pseudomallei | bacteriophages | antimicrobial susceptibility | efflux pump | infection | biothreat

The Search for *Burkholderia pseudomallei* Bacteriophages that Mediate Antimicrobial Susceptibility through Efflux Pump Dependent Infection

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Burkholderia pseudomallei is a significant public health and biothreat pathogen that is recognized as a potential Weapon of Mass Destruction (WMD) due to its resistance to treatment. Moreover, its natural prevalence means that it can infect deployed Warfighters by acquisition from natural environments or by nefarious means. Antibiotic treatment of *B. pseudomallei* infections and post-exposure prophylaxis are hampered by the bacterium's intrinsic and acquired antimicrobial resistance (AMR). Efflux pumps, specifically those of the resistance nodulation cell division (RND) family, are major AMR factors and the sole known multidrug resistance (MDR) determinants in *B. pseudomallei*. These pumps often compromise the therapeutic use of current drugs and those in pre-clinical or clinical development. As such, novel strategies aimed at disarming efflux pumps hold much promise for greatly improving existing but rather limited therapeutic regimens, or perhaps even affording new treatment strategies, thus providing novel Counter WMD (C-WMD) approaches. The objective of our project is to examine the feasibility of exploiting *B. pseudomallei*-specific bacteriophages that use surface exposed outer membrane (OM) channel proteins of RND efflux pumps. We anticipate that mutations in the efflux pumps enabling evasion of phage infection will potentiate the activity of previously ineffective antibiotics. The overall goal of the initial work was to identify phages that bind to the OM channel proteins - OprA and OprC - of the clinically significant AmrAB-OprA and BpeEF-OprC efflux pumps. This required the construction of isogenic mutants expressing or lacking AmrAB-OprA and mutants expressing or lacking BpeEF-OprC in the Select Agent exempt *B. pseudomallei* strain Bp82. Deletion of the OprA gene alone does not completely eliminate antibiotic efflux activity so efforts were focused on phages that utilize the OprC pump. Also, to eliminate identifying phages that utilize LPS as a receptor, we also generated a LPS deletion strain in the OprC expressing background. This strain was then employed for phage screening and characterization. Soil samples from Thailand, where *B. pseudomallei* is endemic, were seeded with the OprC-pump-utilizing variant to amplify bacteriophage growth before filtration and top agar plaque assays were performed. Of the fifty soil samples seeded, plaques were observed on seven plates. Resulting plaques were further amplified and screened against the OprC-deletion variants to remove phages with non-OprC-binding-specific activity. Twenty-two total plaque samples were cross-screened against these variants, and will be additionally tested with the LPS-deletion variants to further cull the collection before full characterization. Phage growth dynamics are evaluated by monitoring bacterial biomass on a multi-mode plate reader and one-step growth curves to determine burst size. Further characterization will include host-range studies, TEM, and sequencing. The results of this project are expected to advance C-WMD science in that they move forward a novel aspect of phage therapy for *B. pseudomallei* infections that can potentially be developed into a specific therapy immune to traditional AMR mechanisms for infected Warfighters.

Human skin microbiome | coagulase-negative Staphylococcus | skin bacteriophages | Staphylococcus phage resistance | experimental evolution | skin health relationships

Identifying skin bacteriophages of Staphylococcus to determine population and community dynamics

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1. IVES
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Human skin is colonised by diverse microbial communities that are important for skin health. Species of coagulase-negative Staphylococcus are highly abundant and vary across the different skin regions, with S. epidermidis being the most frequent. We seek to investigate how the skin virome influences the dynamics of staphylococcal populations in bacterial communities of the skin microbiome, with focus on skin bacteriophages infecting Staphylococcus spp. We collected swabs from >80 healthy volunteers across different skin sites to isolate cutaneous phages that infect different Staphylococcus spp. From the swabs we isolated, purified and sequenced the genomes of 42 phages that infect 8 different Staphylococcus species, including multiple isolates of a novel phage. We assessed qualitatively the degree of phage resistance using a wide host range of 138 Staphylococcus strains of the 8 species. Among the coagulase-negative staphylococci tested, S. hominis exhibits broad resistance to phage infection. We hypothesise that S. hominis encodes pathways to limit phage infection and have explored its mechanisms using experimental evolution by selecting for phage resistance. We also determined that S. aureus was resistant to every phage isolated from the skin that infects coagulase- negative staphylococci. The phages that infect staphylococci will inform our studies of their potential contributions to skin population dynamics and dysbiosis and will enable interrogation of metagenomic datasets to explore relationships with skin health.

Bacteriophage genomes | compact genome architectures | overlapping regulatory elements | M13 phage | Phage and Robotics Assisted Near Continuous Evolution | genome streamlining evolution

Exploring Phage Genome Compaction Using Automated Experimental Evolution

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Bacteriophage genomes can be incredibly compact, making them a useful model for exploring highly optimized genome architectures. This compact nature is often achieved through overlapping open reading frames and regulatory elements of adjacent genes. One instance is the genome of the widely used phage M13, in which 9 of the 10 genes are regulated by ribosomal binding sites (RBS) located internal to the 3' end of the upstream gene. The overlapping functions of this sequence bring into question the tradeoff between optimal RBS sequence identity, which regulates gene expression levels, and optimal C-terminal sequence identity, which dictates protein structure. To investigate this tradeoff, we have created an M13 library containing sequence variants at 7 known internal RBS. Using Phage and Robotics Assisted Near Continuous Evolution (PRANCE), we can evolve this phage library under mutagenic continuous culture conditions maintained by liquid handling automation. The PRANCE platform allows 48 replicate evolving communities to be maintained indefinitely, each encompassing thousands of generations of phage. Using long-read sequencing to track the evolving phage populations over time allows identification of the highest fitness variants and examination of how the initial library evolves to optimize genetic code for dual regulatory and structural functions. Through tracking phage evolution with exceptionally high throughput and resolution, we are well poised to elucidate the evolutionary mechanisms of genome streamlining in phages.

Pseudomonas aeruginosa | phage therapy | antimicrobial resistance | integrases | Podophages

Isolation and genomic characterization of local lysogenic and lytic podophages against *Pseudomonas* aeruginosa

Jorrico Suelto II*, Edelca Panisales, Timothy Mendoza, Ron Leonard Dy

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Pseudomonas aeruginosa is a ubiquitous and opportunistic bacteria known to inhabit diverse environments and has been frequently associated with multidrug-resistant infections in immunocompromised individuals. In nature, bacteriophages serve as natural predators of *P. aeruginosa* and growing interest in phages has risen over the years not only for their potential to combat antimicrobial resistance but also to serve as tools for studying *P. aeruginosa*. In this study, 20 phages against *P. aeruginosa* were isolated from wastewater from the Philippines. All phages were evaluated and four were selected for further analysis due to their broad host range, unique and uniform plaque morphology, high efficiency of plating, and unique restriction digest profiles which altogether maximize the probability that each phage is unique before sequencing. Phages S4C1C (PK), S6C4V (PO), and S9C2V (PT) belonged to the genus *Jamesmcgillvirus*, whereas S7C1C (PE) belonged to the genus *Kochitakasuvirus*. Both genera are members of the Podoviridae family of phages and are among the poorly represented phages in genome databases with only 2 representative phages known according to the International Committee on Taxonomy of Viruses (ICTV). The whole genomes of the four podophages have high degrees of similarities (~95%) to their closest homologue and follow similar genome organizations and sizes. Although both phages S4C1C (PK) and S6C4V (PO) do not appear to possess integrases or recombinases in their genome, both caused lysogenic conversion of *P. aeruginosa* suggesting they are likely temperate phages. These phages may be developed as genetic tools or engineered for targeted genetic delivery of genes in *P. aeruginosa.* Whereas, phages S7C1C (PE) and S9C2V (PT) are better candidates for use as antimicrobials as these were found to be strictly virulent and had no annotated integrases on their genomes. Finally, this study exemplifies the abundance of phages in the Philippines which may be employed as genetic tools or developed as antimicrobial agents of *P. aeruginosa*.

Acinetobacter baumannii | myoviruses | cytidine arabinosylation | DNA modifications | Phage biology

DNA hyper modifications: extreme cytidine arabinosylation in *Acinetobacter baumannii* T4-like phages

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Acinetobacter baumannii is a Gram-negative bacterium known for its intrinsic pan-antibiotic resistance and ability to cause healthcare-associated infections. A potential treatment option rapidly gaining interest is “phage therapy”. We have recently isolated three novel myoviruses vB_AbaM-DLP1, vB_AbaM-DLP2, and vB_AbaM-DLP3 from sewage samples using the *A. baumannii* strain AB5075. Host range analysis of these phages against 107 *A. baumannii* clinical strains shows a limited host range, with 15, 21, and 19 isolates sensitive to DLP1, DLP2 and DLP3, respectively. All phages exhibit large burst sizes of approximately 250 PFU/cell, with short latency periods of 15-20 min. A restriction fragment length polymorphism analysis using a panel of 16 enzymes shows that all genomes are highly resistant to digestion. Only NdeI can cut the DNA when incubated for the maximum allowable time, suggesting significant steric hindrance of the enzyme. More significantly, mass spectrometry of the nucleosides revealed a hypermodified deoxycytidine with a molecular weight of 640 kDa, nearly 2.6 times the size of a normal cytidine. NMR analysis confirmed significant modifications to the deoxycytidine with three arabinose moieties, producing the structure β-D-Araf-5-β-D-Araf-3-α-D-Araf-5-deoxycytidine. Further investigation into the genomes of these *A. baumannii* T4-like phages reveals a conserved region with eight genes encoding enzymes potentially involved in the DNA modification pathway. CRISPR interference experiments are underway to target these genes during host infection to elucidate the enzymes responsible for this significant DNA modification. The identification of this unique, previously unreported DNA modification provides insight into the types of DNA modifications bacteriophages employ to protect their genomes from degradation caused by resident restriction systems of the host.

Staphylococcus aureus | bacteriophage therapy | antibiotic-resistant infections | phages

The Isolation, Characterization, and Optimization of Bacteriophages Targeting Clinical Isolates of *Staphylocccus aureus*.

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1. New York Institute of Technology, Department of Biological Sciences

Staphylococcus aureus is one of the leading causes of global mortality due to bacterial infection. Antibiotic-resistant forms such as methicillin-resistant *S. aureus* (MRSA) are more difficult to treat, which poses an increased risk of complications to patients. Multiple organizations predict that by 2050, antibiotic-resistant bacterial infections could cause 10 million deaths a year. Therefore, developing effective treatments for antibiotic-resistant infections is urgently needed. Recent evidence shows that bacteriophage (phages, viruses that infect bacteria) therapy may represent a viable and successful solution to this emerging crisis. We describe the isolation, characterization, and optimization of bacteriophages capable of infecting a clinical isolate of MRSA from a pediatric patient suffering from osteomyelitis. Although isolating bacteriophages from environmental sources capable of infecting the clinical strain was challenging, ultimately, we succeeded in finding two bacteriophages from commercial bacteriophage cocktails. The two isolated bacteriophages formed plaques efficiently on the clinical isolate at 30 °C, but not at 37 °C. Through repetitive rounds of culturing at 37 °C, we isolated a mutant bacteriophage that infects the clinical isolate efficiently at 37 °C. Future experiments will utilize the optimized bacteriophage to study its therapeutic potential in a mouse-infection model of osteomyelitis.

built environment | microbiome | bacteriophage | biofilm | human health

Phage host interactions in indoor biofilms

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The average American spends 93% of their time in built environments, almost 70% of that is in their place of residence. Human health and well-being are intrinsically tied to the quality of our personal environments and the microbiomes that populate them. Conversely, the built environment microbiome is seeded, formed, pressured and re-shaped by occupant behavior, cleaning, personal hygiene and food choices, as well as geographic location and variability in infrastructure. This ultimately leads to the development of unique microbiomes at different interfaces in our homes. Here, we focus on the presence of viruses in household biofilms, specifically in showerheads and on toothbrushes. Bacteriophage, viruses that infect bacteria with high host specificity, have been shown to drive microbial community structure and function through host infection and horizontal gene transfer in environmental systems. Due to the dynamic environment, with extreme temperature changes, periods of wetting/drying and exposure to hygiene/cleaning products, in addition to low biomass and transient nature of indoor microbiomes, we hypothesize that phage host infection in these unique built environments are different from environmental biofilm interactions. We chose to first approach the hypothesis using metagenomics, querying 34 toothbrush and 92 showerhead metagenomes. Representative of biofilms in the built environment, these interfaces demonstrate distinct levels of occupant interaction. We take an assembly-based approach to identify bacterial and viral genomes and then link virus to host through CRISPR spacers, k-mer identity and prophage integration. Preliminary analysis has identified 4501 low quality 161 medium quality, 62 high quality, and 31 complete circular scaffolds in 92 showerheads, and 3248 low quality, 205 medium quality, 101 high quality, and 25 complete scaffolds on 34 toothbrushes. Statistical analyses were used to determine prevalence and diversity of phage taxa and lifestyles, both lytic and lysogenic. Phage host pairing software identified 1978 potential phage host matchings in showerheads and 3125 in toothbrushes. Current work is focused on parsing high-quality phage contigs and host matches. Next, we plan to compare our findings against the drinking water virome and environmental biofilm viromes using the IMG/VR database. The findings from this analysis will improve our understanding of potential drivers of community formation in biofilms within the built environment. Determining community structure and dynamics of the built environment microbiome enhances our understanding of its relationship to human health. This study will also inform more viral focused sequencing efforts, deeper sequencing, and virus focused methods for DNA extraction, aimed at understanding viral impact on the microbiome in the built environment.

Africa Asia low- and middle-income countries LMIC capacity building

Facilitating Phage Applications in Africa and Asia

Tobi Elaine Nagel

1. Phages for Global Health

Phages for Global Health is a non-profit organization that facilitates the application of phages to combat antimicrobial resistance (AMR) in Africa and Asia, where 90% of the deaths from AMR are expected to occur. Our projects are accomplished through partnerships with >60 collaborators from over 20 countries. The four main pillars of our work include:

(1) Capacity Building

We have delivered 5 workshops for scientists from East Africa, West Africa and Southeast Asia, teaching phage isolation and characterization and how phages might be utilized in people, animals, crops and aquaculture.

(2) Product Development Projects

We have brought together international, multidisciplinary teams to develop:

- *Campylobacter* phages for poultry meat decontamination (Kenya)

- *Vibrio* phages for preventing transmission of cholera between family members (Democratic Republic of Congo and Bangladesh)

- *Mycobacterium ulcerans* phages for treating Buruli ulcer, a neglected tropical skin disease (Benin)

- *Salmonella* innolysins (country TBD)

(3) Advocating for the Establishment of National Phage Banks

We are bringing together global phage experts to draft guidelines on how phage banks might be managed so as to facilitate sharing of phages across international borders. The long-term intention is to have guidelines that scientists in Africa and Asia may utilize as they establish phage banks in their countries.

(4) Regulatory

As part of a Fulbright Global Scholar project during 2023-2024, Tobi will meet with national leaders in Kenya, Uganda and Malaysia to discuss how phage-based drugs are being regulated elsewhere and brainstorm about how phages might be regulated in those 3 countries.

methylation bacteria phage DNA modification defence systems

The role of methylation in the arms race between bacteria and phage

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Bacteria have developed a wide range of defence systems in response to the intense pressure from bacteriophages (or phages) to combat phage infections. Among these defence systems, restriction-modification (RM) systems are the most abundant and widespread. RM systems operate by modifying DNA to either prevent or allow DNA restriction, serving as a prime example of the critical role of methylation in the ongoing arms race between bacteria and phage. Interestingly, many recently discovered defence systems have DNA binding and cleavage capacities. Novel systems like Dnd, Ssp, DISARM and BREX harbour methyltransferases and distinguish self from non-self DNA through methylation, similar to RM systems. Other systems like Gabija and Druantia provide varying levels of protection against phages with different methylation patterns, even though no methylation-related genes are predicted within their operons. To systematically investigate the role of methylation on phage infection, we used long-read sequencing to determine the methylation patterns in the *Escherichia coli* Reference Collection (ECOR) and phages produced using hosts with diverse methylation backgrounds. By examining the shift in the phage host range between different methylation backgrounds and its correlation with bacterial defence systems, we shed light on the role of methylation in phage infections, and gain valuable insights into the selection of suitable production hosts for phage therapy applications.

Klebsiella pneumoniae | phages | infections | hypervirulent strains | ESKAPE pathogens

An open collection of phages targeting *Klebsiella* spp.

Daniela Rothschild-Rodriguez [1*], Morgen Hedges [1], Claire King [1], Sophie Lawson [2], Matthew Wand [2], Mark Sutton [2], Franklin L. Nobrega [1]

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Klebsiella pneumoniae is an encapsulated Gram-negative pathogen most commonly known for its involvement in nosocomial and community-acquired infections. These infections can range from pneumonia, urinary tract, and soft tissue infections to more severe conditions such as septicaemia, liver abscess, and sepsis, particularly for the hypervirulent strains. *K. pneumoniae* is also recognised as one of the six “ESKAPE” pathogens in need for the development of new antimicrobials. Recently, gut microbial colonization by this bacteria has been linked to inflammatory diseases like inflammatory bowel diseases and primary sclerosing cholangitis. As a result, bacterio(phages) have become increasingly relevant as potent therapeutics for treating *K. pneumoniae* infections and gut colonisations. However, the high variability of the *Klebsiella* capsule, with up to 79 known capsular (K) types to date, poses a significant challenge for receptor-mediated strategies targeting this pathogen (e.g. vaccine developments and phage therapy). Thus, it is crucial to understand phage-mediated interactions to ensure successful therapeutic applications. Unfortunately, this understanding is currently limited, partly due to the lack of a representative and accessible collection of *Klebsiella* phages. To this end, our aim was to establish an open-source collection of phages targeting *Klebsiella* spp. that will be accessible for collaborative and additive research via an online platform. In the initial phase of this project, we isolated and fully characterised 47 phages (the Roth collection) that can target a variety of *Klebsiella spp.* including some of the most clinically relevant clonal groups (e.g. ST258, ST15, ST14, ST23, ST17, ST86, ST11) and capsular types (e.g. K1 and K2). The phage stocks and associated data will be freely available, and users of the collection are expected to contribute and share data, such as host range information and case/clinical studies. We envision that this open-source collection will significantly contribute to expand our understanding of phage-mediated interactions to facilitate phage therapy applications.

T1 Phage | Population Dynamics | E. coli

Determining the Relationship between the carbon source for Escherichia coli and T1 Phage Growth Rate

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Bacteria play a huge role in the human and natural world, for richer and for poorer, with their most well-known role being a pathogen. With the gradual reduction in antimicrobial efficacy, the promise of phage as a potential solution has gathered attention. For phage therapy to be successful it must be applicable in a wide range of environments.

In each of these environments, phage relies on bacterial ribosomes to takeover the bacterium. In exponential growth in environments with different quality of a carbon source to be metabolised, bacteria must find the optimal number of ribosomes to allow it to reproduce, and this number has been shown to alter linearly with the growth rate in each environment.

Here I have investigated how the bacteria-bacteriophage system dynamics depends on the carbon source being used by the bacteria. Escherichia coli, grown in a range of media with various carbon sources were exposed to T1 bacteriophage. In all cases the bacteria developed resistance to the phage at long time. However, the population dynamics of the system both post and during infection varied with both carbon source and the number of phage added. Through initial fitting of the phage dynamics by using a simple model, it appeared that the growth rate of phage was uncorrelated with the growth rate of the bacteria. Then, with a more sophisticated model we probe which factors are dominant in the growth of a phage population as well as whether there are multi-stage processes dominant within the dynamics.

These results show the relationship between carbon source and the progression of a bacterial populations response. These results contribute to the understanding of the dominance and structure of factors in the growth of phage which could result in significant differences between the rates of death and resistance development in vivo and in vitro due to differing environmental conditions.

TB tuberculosis Mycobacterium tuberculosis anti-phage humoral immunity preclinical mouse model ELISA methodology

High throughput ELISA methodology for evaluating anti-phage humoral immunity in the preclinical mouse model of tuberculosis

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For five of the last ten years tuberculosis (TB) disease, caused by active infection with *Mycobacterium tuberculosis* (M.tb), has been the leading cause of death globally. Therapies designed to complement drug treatment or disrupt M.tb transmission are urgently needed while next generation vaccines and drugs are being evaluated. Mycobacteriophages are examples of readily abundant biologics that are bactericidal against M.tb and can help fill this immediate gap. However, it has been reported that repeated intravenous (i.v.) phage treatment for other mycobacterial infections can induce a host humoral immune response that may interfere with therapy. Here we aimed to develop a total IgG, IgM and IgA high-throughput ELISA to evaluate the magnitude of anti-phage antibody responses in a preclinical C57BL/6 therapeutic mouse model. We selected representative anti-M.tb phages Muddy HRM*N0157-2* (Muddy) and FionnbharthΔ*45*Δ*47* (Fionnbharth), which have been shown to have lytic activity against M.tb. Cohorts of mice were given 10^8 pfu/mL of phage in 200µl by i.v. weekly for four weeks and serum was collected weekly for five weeks. To measure serum antibody levels, 384 well ELISA plates were coated with 10^8 pfu/well of phage in either traditional coating buffer or phage buffer and incubated overnight at 4°C. Serum samples were initially diluted 1:10, added to the first column of the plate and subsequently diluted 1:5 in diluent for a 12-point dilution series. After incubation and washing, plates were treated with HRP-conjugated anti-mouse total IgG, IgM or IgA and incubated for one hour at room temperature in the dark. Plates were then washed and TMB Sureblue Peroxidase Substrate was added. Once the blue color change appeared to have reached a saturation point, the reaction was quenched by adding 25 µl of 1N H2SO4 simultaneously to each well of the plate. Absorbances were read at 450nm and 570nm and Optical Density values are calculated using 450nm-570nm. Control (untreated serum) samples were used to set cutoffs and determine endpoint titers (EPTs). We observed that both traditional ELISA coating buffer and phage buffer coating resulted in the same EPTs and either can be used confidently in this assay. For total IgG responses against Muddy and Fionnbharth cohorts, we observed a time and dose dependent increase in anti-phage EPTs in serum from matched i.v. treated animals where peak responses were recorded at day 28. In addition, we did not observe measurable EPT responses to the opposite phage, suggesting these responses are phage-specific. We confirmed a very low anti-phage IgA response in the serum as expected. The serum anti-phage IgM responses were non-discernable due to high background and this suite of assays requires further optimization before deploying. In summary, we have successfully developed a reproducible and sensitive ELISA for the detection of anti-Muddy or anti-Fionnbharth mouse total IgG and IgA responses from serum samples. This methodology required no adaptation between phages for use and serves as a platform to further test anti-phage humoral immunity in the mouse model in the context of repeated deliveries and different routes of delivery for treatment of tuberculosis.

Phage therapy | Antagonism | Antibiotics | Bacteriophage therapy

Impacts of meropenem and tobramycin on infection activities of *Pseudomonas aeruginosa* phages

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In the clinic, phage therapy often is used in combination with antibiotics, including against *Pseudomonas aeruginosa* infections. Antibiotics, however, interfere with critical bacterial functions, including DNA replication and protein synthesis, that are required for phage infection success. Nevertheless, the impact of antibiotics on phage replication is not commonly determined prior to phage therapies. Here we use an optical density-based 'lysis profile' assay to assess the impact of two classes of antibiotics – meropenem, and tobramycin – on the bactericidal, bacteriolytic, and virion-production activities of *P. aeruginosa* phages PEV2 and fKMV. This is a rapid, high-throughput assay that provides results of phage-antibiotic interactions within a few hours, e.g., as could be employed prior to the start of phage-antibiotic combination treatments. The antibacterial activities of phages and antibiotics in combination were found to be greater than those of phages or antibiotics alone. Tobramycin, however, substantially interfered with phage bacteriolytic and virion-production activities, even at minimum inhibitory concentrations (MICs). Meropenem, by contrast, had no or minimal impact including at clinically relevant concentrations (up to 27× MIC). We corroborated these results by more traditional measurements: colony forming units, plaque forming units, and one-step growth experiments. Furthermore, we analyzed activity of mono- and combination therapies against biofilm. Collectively, this indicates that meropenem may be minimally antagonistic in phage therapy co-treatments of *P. aeruginosa* infections. More generally, our results point to a prospect of rapid, routine testing of antibiotic antagonism of phage infection activities prior to the initiation of phage treatments.

polylysogeny phage fitness host C. rodentium in vitro

Understanding polylysogeny: Phage phiNP contributes to the fitness of its host, *C. rodentium*, *in vitro*

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Understanding polylysogeny: Phage phiNP contributes to the fitness of its host, *C. rodentium*, *in vitro*

Citrobacter rodentium is a murine host adapted member of the attaching and effacing (A/E) family of pathogens, and is an important model in the study of the closely related diarrheal pathogens, Enteropathogenic *Escherichia coli* (EPEC) and Enterohaemorrhagic *E. coli* (EHEC). Much complexity of this model remains unknown, including the interaction between phages and the virulence of *C.rodentium*. Previous research has shown that *C.rodentium* DBS100 contains 10 prophages, while only two have been shown to be active temperate phages. While most intestinal bacteria are lysogens, the role of multiple phage carriage in host fitness and colonization is largely unstudied. Here we 1) examine the lifecycle of phiNP as a potential phagemid, 2) begin understanding the *in vitro* role of this phage during bacterial competition and 3) the impact of polylysogenic interactions on host colonization *in vitro*. We are studying the contribution of these phage to the competitiveness of their host, DBS100. We hypothesize a competitive advantage of the polylysogen compared to both the mono and nonlysogen counterparts *in vitro*, given the presence of virulence genes and interaction between phages. Polylysogeny, has been shown to mediate bacteria–bacteria competition, with this in mind we aim to understand how phages phiNP and Shae_phiSM contribute to the polylysogenic nature of DBS100. We show that phiNP enters a lysogenic lifecycle that is maintained during the hosts stationary phase, correlating with the drastic reduction in host fitness observed in the phiNP nonlysogen. Another interaction being studied is the recombination that occurs between Shae_phiSM a P2-like temperate phage and the a putative P4-like satellite, CRPr20, that is required for virion production. This P2-P4 interaction may be used as a model to understand one role of polylysogeny and the importance of phage interactions for the colonization of *C.rodentium* DBS100.

temperate phage | induction | P2 | SOS pathway | evolution | c-di-GMP-phosphodiesterase

Probing how recurrent stress encounters shape the future response of temperate phage P2

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Temperate phages can be seen as built-in architects, having a tremendous capacity to shape the ecology and evolution of microbial communities. With their possibility of entering lytic or lysogenic replication, they can either erase specific bacterial lineages or rewire the biology of their bacterial host. Temperate phages are found in at least half of all bacteria composing the gastrointestinal microbiome and thus can influence the microbiome by switching between replication strategies. One example is P2-like phages that infect strains from 127 different host genera, including clinically relevant strains of *Escherichia coli* and *Salmonella*. Thereby, the SOS response pathway induces a subpopulation of those P2 phages to switch from lysogenic to lytic replication. The SOS pathway can be activated by several stimuli present throughout the intestine. Therefore, P2 phages are recurrently exposed to induction stimuli in the gut, which opens the question of whether temperate phage P2 adjusts the lytic and lysogenic decision-making threshold because of recurrent stress exposure. We can show that induced P2 can infect and re-integrate into the genome of an *E. coli* mutant genetically cured of its phage. During such autologous infections, we revealed that the number of phages entering lysogenic replication decreases with each round of induction and infection. Furthermore, induced P2 selects for hosts carrying a mutation in the *pdeI* gene encoding for a c-di-GMP-phosphodiesterase, which is involved in regulating the c-di-GMP concentration. C-di-GMP is a bacterial secondary messenger that controls core bacterial processes like growth, metabolism, and stress response. We combine synthetic biology and experimental evolution to test if previous stress encounters prime future phages' reactions by evolving replication strategies or selecting a host subpopulation adjusted to endure in a challenging environment, thus securing phage persistence in the microbiome and modulating the microbial community.

T5 DNA ligase genome engineering

Structure and function of the unique split DNA ligase encoded by T5-like bacteriophages

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In all forms of life, DNA ligase enzymes are essential for joining strands of DNA when new DNA is being made or when damaged DNA is being repaired. These enzymes fall into two classes dependent on their requirement for either ATP or NAD+ as a co-factor. Most bacteriophage genomes sequenced to date do not encode a DNA ligase; these phages rely on the activity of the host cell ligase for lytic infection. The T5-like phages (*Demerecviridae*) in contrast encode a ligase that is essential for phage infection. This NAD+ -dependent ligase has a highly unusual structure: the enzyme is split into two separate polypeptides encoded by distinct non-overlapping ORFs. This structural organisation - which is unprecedented in DNA ligase biology - is an evolutionarily conserved feature of all T5-like phages, implying that having a split DNA ligase is important for phage fitness. We have initiated a study of the T5-like split DNA ligase, aiming to better understand its structure, its biological function, and the evolutionary rationale for splitting the enzyme into two parts. In particular, we aim to determine whether the existence of the split ligase could be related to the presence of single-strand DNA nicks in the packaged phage genome. We have determined the crystal structure of the split ligase encoded by *Providencia rettgeri* phage vB_PreS_PR1 (PR1) bound to nicked substrate DNA, offering insight into how the two subunits come together to form an active enzyme, and have embarked on a programme of genome engineering of phage T5 using CRISPR/Cas methodologies to ask whether the split is essential for ligase function. The results of these studies will be described.

Pseudomonas aeruginosa | bacteriophage | silver nanoparticles | biofilms | antibiotic resistance

The Combined Application of Bacteriophage and Silver Nanoparticles to Combat *Pseudomonas aeruginosa* Biofilms

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Pseudomonas aeruginosa is an opportunistic pathogen that causes >50,000 infections in hospitalized immunocompromised patients and >2,500 deaths in the United States annually. With the increasing rise of antibiotic resistance acquired by this bacterium, the World Health Organization (WHO) listed *P. aeruginosa* as a critical Global Priority Pathogen (GPP) in need of new therapeutic methods. Hence, the main objectives of this research are to isolate and characterize *P. aeruginosa* lytic phages and to investigate their combined application with silver nanoparticles to combat *P. aeruginosa* biofilms.

Six sewage water samples were enriched for phages using nine *Pseudomonas spp* strains. Twenty bacteriophages were isolated based on plaque morphology and tested against 38 *P. aeruginosa* clinical isolates at a multiplicity of infection (MOI) of 1. Five phages were found to have a broad host range profile and inhibited the growth of multiple *P. aeruginosa* clinical isolates. Subsequent screening experiments were conducted to determine the phages' virulence on all 38 strains and one phage showed high virulence being able to infect 22/38 strains efficiently. *P. aeruginosa* strain PA14 was selected for all downstream phage characterization and host interaction experiments, such as adsorption efficiency, one-step growth curve, bacteriophage insensitive mutant (BIM) frequency, minimum inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC) experiments. One single phage, Chandler, had a comparable biocontrol efficacy against planktonic PA14 cells versus a cocktail of three phages. Therefore, only Chandler was chosen for downstream biofilm assay experiments. During genomic analysis it was discovered that Chandler belongs to the family Myoviridae, has a genome size of approximately 66 kbp and contains no lysogenic genes. Through adsorption kinetics experiments, it was determined that Chandler has a large burst size of 144 and latent period of 30 minutes. Silver nanoparticles sensitivity experiment revealed that the chosen phage is not affected by silver nanoparticles even after being exposed to silver nanoparticles for 24 hours at 200 RPM at a concentration of 10 ng/mL and 10 7 PFU/mL. The checkerboard and MBEC assays revealed that when a combined application of phage and silver nanoparticles are used against PA14 biofilms at a concentration of 10 9 PFU/mL and 10 ng/mL respectfully, had comparable efficacy in biofilm degradation as 10 3 PFU/mL and 10 ng/mL. There was a 75% reduction in biofilm and the biofilm was only able to recover to 25% of its original biomass when treated with both phage and silver nanoparticles together. Biofilm assays are quite variable, and the results indicate that there is not a notable synergistic effect. However, the reduction in biofilm biomass and viability shows promising results for downstream applications and future experiments. Thus, these findings indicate that when using a biological entity and a chemical compound such as silver nanoparticles can present some challenges and the dynamics may be more complex than previously expected.

anti-phage defence | anti-plasmid defence | evolution and ecology | HGT

4242 – the answer to life, the universe, and everything? Integrating evolutionary and molecular microbiology to understand plasmid costs and phage defence associated with a putative Type IV restriction-modification system

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Horizontal gene transfer (HGT), often facilitated by mobile genetic elements (MGE), is an essential component of bacterial evolution and ecology. HGT facilitates the spread of significant traits including antimicrobial resistance (AMR). However, incoming genes can introduce genomic conflict. The basis of such conflict can lie in specific gene-gene interactions, but the mechanistic basis, and whether such conflicts are host specific, is not clear.

There is an intrinsic, universal evolutionary pressure for bacteria to defend themselves against such invasive, foreign DNA. This has led to bacteria developing ways to protect themselves against MGEs such as phage and plasmids by encoding a diverse range of defence systems. The anti-phage defence field is a rapidly emerging and growing discipline of study with the discovery of dozens of novel systems reported upon within the last few years. Crucially, the systems we are currently aware of are thought to be the very tip of the iceberg and it is anticipated that the full antiphage weaponry of bacteria will likely include hundreds, if not thousands, of diverse, mobile defence systems. Furthermore, recent work is beginning to shed light on the ability of phage and plasmids to counter-defend against these systems through the carriage of what has been termed 'anti-defence'- systems.

In order to successfully combat the global and complex issue of AMR, it is imperative that we better understand the conflict between phage and plasmid defences encoded by bacteria and their mobile genetic elements.

Several naturally-occurring mercury resistance 'pQBR' plasmids impose significant costs to *Pseudomonas fluorescens* SBW25, and previous work indicates the principal source of conflict is hypothetical DUF262 domain-containing chromosomal protein PFLU4242 (4242). DUF262 is predicted to act as an NTPase, and has been identified within several recently discovered novel anti-phage systems including BrxU, Dazbog and PD-T4-2. 4242 has been identified as a putative Type IV restriction-modification (RM) system that may play a role in anti-MGE defence; 4242-pQBR interactions are therefore an exemplary model of MGE conflict. 4242's mechanism of action is unknown, as is whether 4242-pQBR conflict is genetic background specific.

We analysed 4242 homologue distribution across diverse species, and found that it often resides within Defence Islands and is widely distributed via HGT. Given this genomic co-localisation of 4242-like proteins with other Defence Island elements, we hypothesise that 4242-like proteins are a genome defence mechanism. To explore this further, we cloned 4242 and a naturally-arising inactive mutant into *Escherichia coli* DH5-alpha and challenged these isogenic strains with a panel of coliphage. 4242 failed to show activity against glc-5hmC, 5mC, or 5hmC modifications despite being a homologue of the well characterised GmrSD and BrxU anti-phage defence systems which are known to provide robust protection against T-even phage.

To understand 4242's physiological activity and ecological function, we next expressed 4242 and the naturally-arising inactive mutant in other *Pseudomonas* species. Our results help answer whether 4242-pQBR conflict is specific to *P. fluorescens* SBW25, or might operate across the *Pseudomonas* pangenome. Our data provides better understanding of MGE/host dynamics and explores the poorly understood trade-off between openness to HGT and genome defence: a key mechanism determining genome content, adaptive capacity, and pangenome structure.

Our current work focuses on a 4242-homologue found in *Salmonella enterica* serotype Newport, in order to better understand the anti-phage defence capabilities of such Type IV RM systems in a clinically relevant strain that is rapidly emerging as a pathogen in both animals and humans. We also seek to investigate conserved Defence Island hotspots in this serovar.

endolysin | live biotherapeutic products | purity | potency

Characterization of Lactobacilli phage Endolysins functional domains For Use In Biotechnological Applications in Live Biotherapeutic Product Development

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Phage endolysin specific binding characteristics and killing activity support their potential use in biotechnological applications, such as potency and purity testing of live biotherapeutic products (LBP). LBPs contain live organisms, such as Lactic acid bacteria (LAB), and are intended for use as drugs. Our approach is to use endolysin cell wall binding domains (CBD) for LBP potency assays and endolysin killing activity for purity assays. CBDs of 5 lactobacilli phage lysins, CL1, Jlb1, Lj965, LL-h, and ϕJB were characterized. They exhibited different binding to 27 LAB strains and were found to bind peptidoglycan or surface polymers. Flow Cytometry based on CBD binding was used to enumerate viable counts of two strains in mixture. CL1-lys, jlb1-lys, and ϕJB-lys and their EADs exhibited cell wall digestive activity and lytic activity against LAB. Jlb1-EAD and ϕJB-EAD were more sensitive than their respective hololysins to buffer pH and NaCl concentration changes. The ϕJB-EAD exhibited stronger lytic activity than ϕJB-lys, possibly due to ϕJB-CBD-mediated sequestration of ϕJB-lys by cell debris. CBD multiplex assays indicate that these proteins may be useful LBP potency reagents and the lytic activity suggests that CL1-lys, jlb1-lys, and ΦJB-lys and their EDs are good candidates for LBP purity reagent development.

Phage resistance | RNA-seq | transcriptomics | MRSA

The effect of *Staphylococcus aureus rpoC* mutations that confer phage resistance on the transcriptional program of phage K

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To better understand host-phage interactions and the genetic bases of phage resistance in a model system relevant to potential phage therapy, we isolated several spontaneous mutants of the USA300 *S. aureus* clinical isolate NRS384 that were resistant to phage K. Six of these had a single missense mutation in the host *rpoC* gene, which encodes the RNA polymerase beta prime subunit. To examine the hypothesis that the mutations in the host RNA polymerase affect the transcription of phage genes, we performed RNA-seq analysis on total RNA samples collected from NRS384 wild-type (WT) and $rpoC_{G26D}$ mutant cultures infected with phage K, at different time points post-infection. Infection of the WT host led to a steady increase of phage transcription relative to the host. Our analysis allowed us to define 55 transcriptional units and to define different early, middle, and late phage genes based on their temporal expression patterns. An examination of predicted promoter sequences revealed that those associated with late gene expression lacked a conserved -35 region, suggesting the involvement of a phage-encoded factor in the transcription of those genes. Infection of the $rpoC_{G26D}$ mutant host led to a transcriptional pattern that was similar to the WT at early time points. However, beginning at 20 minutes post-infection, transcription of late genes, such as phage structural genes and host lysis genes, was severely reduced. Our data indicate that the $rpoC_{G26D}$ mutant is deficient in proceeding to late phage gene expression, thus resulting in a failed infection cycle for phage K. In addition to providing a detailed view of the global transcriptional landscape of phage K throughout the infection cycle, these studies can begin to inform our studies into the mechanistic bases of phage K's control of its transcriptional program.

Salmonella foodborne pathogens bacteriophages biocontrol agents antibiotic profile phage vB_Sal_ZC10

Phage vB_Sal_ZC-10; an innovative approach to control *Salmonella* in foods

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(Background): *Salmonella* spp. are significant foodborne pathogens that pose a serious risk to public health. These bacteria are commonly associated with contaminated food, particularly raw or undercooked poultry, eggs, and dairy products. *Salmonella* infections can lead to gastroenteritis, fever, and in severe cases, hospitalization and even death. Bacteriophages, which are viruses that specifically target and kill bacteria, have emerged as a promising strategy to control foodborne contaminants. They offer a natural and environmentally friendly alternative to traditional antimicrobial agents. By employing bacteriophages as biocontrol agents, the risk of *Salmonella* transmission through food can be effectively reduced, enhancing food safety and protecting public health.

(Methods): *Salmonella* host strain ZC_S-30 was isolated and characterized from food samples, and its antibiotic profile and virulence genes were detected. A lytic phage vB_Sal_ZC-10 was isolated from environmental water sewage samples on *Salmonella* host strain ZC_S-30. The isolated phage underwent purification and amplification, and its molecular DNA was assessed using Pulsed Field Gel Electrophoresis (PFGE). Additionally, Transmission Electron Microscopy (TEM) was employed to visualize its structure and morphology, while antibacterial activity against various *Salmonella* spp. hosts was evaluated. Stability tests were conducted, and the entire genome of the phage was sequenced. The efficiency of phage suspension was evaluated in vitro and in milk inoculated with *Salmonella* spp. host strain.

(Results): Phage vB_Sal_ZC10 was found to be belonging to siphoviruses as indicated by the TEM. The PFGE and the phage sequencing estimated the phage genome size of 48 kbp. In addition, the results indicated that phage vB_Sal_ZC10 withstands high stability at different temperatures (-80 up to 80 °C) and pH ranges (pH 3 - 9). For the antibacterial activity, phage vB_Sal_ZC10 exhibited clear zones on its ZC_S-30 bacterial host along with other host strains. In addition, the in vitro results of the time killing tests showed a significant bacterial eradication over different periods of time at varying MOIs (0.001, 0.01, 0.1, 1, 10, 100). Additionally, phage-treated milk inoculated with *Salmonella* host strain showed a biocontrol efficiency by reducing the bacterial titer by a minimum of 2 Log_{10} CFU/ml at MOI of 100 and below the detection limit with MOI of 100,000 over the time of experiment.

Characterization | detoxified Stx-phage prophage | vb_24B_13c | fitness | bacterial host

Characterization of the contributions a detoxified Stx-phage prophage vb_24B_13c makes towards the fitness of its bacterial host.

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Characterization of the contributions a detoxified Stx-phage prophage vb_24B_13c makes towards the fitness of its bacterial host.

Lysogen | Spontaneous Induction | Transcriptomics

Elucidating the impact of prophages on their bacterial hosts during lysogeny through transcriptomics.

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Most bacterial pathogens are lysogens; yet, if the prophage doesn't carry identifiable toxins, little credit is given to any role the prophage may play in the biology of the bacterial host. It is not uncommon to find conservation of all or part of a phage genome across a bacterial species, a phenomenon usually associated with powerful selection of beneficial traits. However, this is rarely attributed to prophages lacking identifiable selective markers/traits, with the majority of prophages carrying genes that are still annotated as hypothetical and consigned to the realm of "phage dark matter". Transcriptomics offers a powerful tool to examine the impact of carrying a prophage or prophages. This has been done before using Shiga toxin encoding prophages, but in this project, we examined the impact of 3 distinct phages of polylysogenic clinical strain of *Pseudomonas* aeruginosa Liverpool Epidemic Strain (LES) that carry no identifiable virulence genes.

LES is a polylysogenic clinical strain harboring five inducible prophages, a probable prophage remnant and a few genomic islands. LES is associated with increased morbidity of chronic *Pseudomonas* infections in the patients with cystic fibrosis. We are investigating the influence of a set of three co-habiting prophages in "multiple combinations", in the model *Pseudomonas aeruginosa* host strain PAO1. Despite strong evidence that their presence in the wild-type *Pseudomonas aeruginosa* host strain PAO1 is associated with a gross increase in virulence of their host, the specific molecular detail is not known. We have optimized growth conditions to favor the lysogenic state and used that condition to further study the gene expression landscape of lysogen under conditions in which prophage is induced and where the prophage is held as stably as possible in the lysogen culture. We validated the induction profile by qRT-PCR of the key phage marker genes from crucial stages of LES phage replication in PAO1 in both conditions. Using the information derived from the q-PCR profiling, we prepared RNA samples for RNA seq library production. We have identified 24 clusters of genes that are differentially regulated by these prophages and combinations of these prophages in the PAO1 background. Our RNA seq data demonstrates that the phage carriage in the lysogeny regulates the expression of many host genes, depending on the type of the phage. Moreover, we show the possible epistatic genetic interactions between two different phages using differentially regulated clusters of genes. Work is ongoing to understand how these genes are regulated and what this means for the biology of the lysogen.

bacteriophages | ssRNA phages | host factors | phage-host interactions

High-throughput mapping of host factors involved in ssRNA bacteriophage infecting pathways

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Bacteriophages are the most abundant biological entities on earth and known to play a crucial role in microbial ecology, physiology, virulence, and nutrient cycling (1). Most studies on bacteriophage abundance, diversity, genetic content, host-specificity, and their effect on microbial communities are currently focused on the limited viral taxa, specifically within the double-strand DNA (dsDNA) phages. However, recent studies on metatranscriptome revealed the actual ubiquity and diversity of non-dsDNA viral particles are largely understudied, and in particular single strand RNA (ssRNA) phages are especially prominent in soil ecosystems (2-6). Despite their importance, shotgun RNA sequencing efforts only provide limited and partial information about these novel ssRNA phages, and especially fail to associate these phages with their host bacteria, infectivity pathways, mechanism of host cell take-over and host lysis. For example, though ssRNA phages have been known to bind conjugative pili elements for infectivity, the knowledge and genetic basis of their infection and resistance has been limited to a couple of canonical phages (7). These early studies primarily isolated phage-resistant host mutants and characterized them using classical genetic approaches. With the recent discovery of tens of thousands of novel ssRNA phages there is a need for high-throughput technologies to characterize ssRNA phage-host interactions. Here, we adopt recently developed two high-throughput genetic technologies (8), Random barcode transposon site sequencing (RB-TnSeq) and Dual-barcoded shotgun expression library sequencing (Dub-seq) to a model *E. coli* strain for discovering host factors, and gene dosage barriers crucial in ssRNA phage infection and bacterial resistance. We resourced a collection of ssRNA phages including three model ssRNA phages, MS2, fr, and Qβ to map genetic landscape important in phage infection. Using genome-wide loss-of-function and gain-of-function genetic technologies, we are able to confirm the importance of conjugative pili elements as well as uncover other host factors playing an important role in ssRNA phage infective pathways. To the best of our knowledge, this is the first such report to systematically characterize non-dsDNA phage-host interactions and opens up an avenue to extend it to the other *E. coli* strains and non-*E. coli* phages.

P. aeruginosa LES | transmissible pathogen | genomic islands | Pseudomonas prophage | LES phage 5 genome | spontaneous induction

LES Phage 5 length, capsidated, amplification, and homologous with other *Pseudomonas* phages.

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P. aeruginosa LES is an opportunistic transmissible pathogen between CF patients, being capable of infecting the lung of non-CF patients and superinfecting patients with other *P. aeruginosa* strains. Inside *P. aeruginosa* LES genome, 20 genomic islands were which included the presence of five active prophages and one prophage-like element. LES φ5 is a temperate and active Pseudomonas-prophage that plays an essential role in host's competitiveness. Seventy-six ORFs are predicted in LES Pseudomonas phage 5. Only 20 ORFs do not have functional prediction (Hypothetical protein). We proved that the total length of LES phage 5 genome is 50,235 bp, and the *attL* and *attR* regions were located at 2,690,327 – 2,690,341 and 2,740,547 – 2,740,561 in the *P. aeruginosa* LESB58 genome, respectively. It was proved that LES phage 5 is complete phage because it encapsidated its DNA.

There are 3 phages share homologous sequences with LES phage 5. Moreover, LES phage 2,3,4 and 5 have spontaneous induction in the log and stationery phase. Differences between the amplification of all LES phages were detected.

Skin microbiome | Staphylococcus | bacteriophages | defence system | population dynamics

Identifying skin bacteriophages of Staphylococcus to determine population and community dynamics

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Human skin is colonised by diverse microbial communities that are important for skin health. Species of coagulase-negative *Staphylococcus* are highly abundant and vary across the different skin regions, with *S. epidermidis* being the most frequent. We seek to investigate how the skin virome influences the dynamics of staphylococcal populations in bacterial communities of the skin microbiome, with focus on skin bacteriophages infecting *Staphylococcus spp*. We collected swabs from >80 healthy volunteers across different skin sites to isolate cutaneous phages that infect different *Staphylococcus* spp. From the swabs we isolated, purified and sequenced the genomes of 42 phages that infect 8 different *Staphylococcus* species, including multiple isolates of a novel phage. We assessed qualitatively the degree of phage resistance using a wide host range of 138 *Staphylococcus* strains of the 8 species. Among the coagulase-negative staphylococci tested, *S. hominis* exhibits broad resistance to phage infection. We hypothesise that *S. hominis* encodes pathways to limit phage infection and have explored its mechanisms using experimental evolution by selecting for phage resistance. We also determined that *S. aureus* was resistant to every phage isolated from the skin that infects coagulase- negative staphylococci. The phages that infect staphylococci will inform our studies of their potential contributions to skin population dynamics and dysbiosis and will enable interrogation of metagenomic datasets to explore relationships with skin health.

Phage amplification assay (PAA) | Antibiotic-resistance | Multiplexed qPCR | Rapid detection

Phage amplification-mediated strategies for the simultaneous detection of foodborne pathogens in foods

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Introduction: Foodborne pathogens could trigger foodborne diseases and have been a major threat to food safety. In the United States, FoodNet has reported more than 25,000 infections each year, resulting in 6000 hospitalizations, and 120 deaths annually from 2016 to 2020. The appearance of antibiotic-resistant bacteria can worsen the condition of infection. Phage-mediated technologies have evolved to be sensitive and rapid methods for the detection of a broad range of pathogens in recent years. Phages are uniquely suited for bacteria detection, which could not only act as probes to specifically recognize viable bacterial cells, but also be signal amplifiers through phage amplification (PAA)-based analysis.

Objective: Hence we proposed PAA-mediated strategies for the quantification of foodborne pathogens as well as the detection of viable antibiotic-resistant strains with high specificity, sensitivity, and accuracy.

Methodology: Our scheme was as follows: (1) Phage with a broad host range and high lysis ability was carefully selected; (2) Then PAA strategy was established and applied for the quantification of foodborne pathogens in foods, as well as assessment of potential antibiotic resistance in different food matrices; (3) Finally, the detection time of this assay was further decreased by combining with a multiplexed qPCR step for the quantification of progeny phage.

Results: Under the optimized conditions, this assay has been successfully applied to quantify viable *Salmonella enterica* and *Staphylococcus aureus* in food matrices such as milk and lettuce, with a detection limit of 10 CFU/mL. When combined with multiplex qPCR assay, PAA-qPCR further reduced the detection time from 6.5 h to 3.5 h, without complicating the DNA extraction or purification process.

Conclusions: The proposed PAA-based multiplex qPCR assay provides an effective and promising strategy for the simultaneous detection of viable bacteria, with rapidity, high sensitivity, and specificity with the ability to distinguish live bacteria.

Phage Therapy | Antibiotic Resistance | Bacteriophage Activity Evaluation | Pseudomonas aeruginosa | Klebsiella pneumoniae | Therapeutic Phage Selection

Phage Activity Enumeration:Comparison of MD/SP, PLC and Tetrazolium Reduction

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Background: Prevention of rapidly spreading of antibiotic-defeating germs is major challenge today. Using of phages as the adjuvants to antimicrobials to address the threat of antibiotic resistance (AR) is very promising. But developing of simple and sensitive methods for phage activity evaluation in a fast manner is limited, because of phages are “living organisms” with high specificity to bacteria.

The optimal management of phage activity evaluation is highly impotent to prevent infections in the way to improve effectiveness of antibiotic-phage synergistic treatment and slow down the resistance development to both bacteria and phage.

Methods. Newly isolated phages of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* from different natural and industrial sewage environments and with sequenced genome were applied to study comparison of results produced from the methods of MD/SP (Multiple Dilutions on Single Plates), PLC (Phage Liquid Culturing) and BTR (Bacterial Tetrazolium Reduction). OmniLog™ system of redox chemistry to automatically measure cell respiration, a marker for bacterial growth.

The phage lytic activity was tested against a large bacterial matrix (103 *P. aeruginosa* and 172 *K. pneumoniae strains*). Efficiency of plating (EOP) was determined using both the agar plaquing and kinetic clearing methods and the results were correlated in parallel with TR and colony-forming reduction (CFR) in a multi-well-spot format.

Results: Based on EOP results, the host coverage of the selected phages of both *P. aeruginosa* and *K. pneumoniae* was about 80% individually. The given approach of phage lytic activity evaluation included pfu/ml and cfu/ml enumerations with correlation-adjustment to TR for a given timepoint. This approach will facilitate further development of phage lytic activity and emerging phage-resistance mutants’ determination, and selection of candidate therapeutic phages.

Oral Disease | Periapical | Pathosis | Periodontitis | Fungi | Microbiome

The Bacteria, Fungi, and Phage Associated with Periapical Pathosis

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Oral diseases akin to gingivitis, periodontitis, caries, pulpitis, and abscesses are common but treatable ailments that have plagued humanity for thousands of years. These diseases, yielding increased pain when chewing, tooth loss, and oral deformations, result from an imbalance in the local microbiota due to improper or inconsistent preventative maintenance. While many of these infections are specific to the surface and immediate subsurface gingival layer, more serious infections can fester deep within these tissues, causing periapical tissue inflammation and degradation Recent studies have demonstrated that around 59% of all root canal procedures result in some degree of periapical pathosis, deeply imbedded within the bone and difficult to access for treatment. Periapical lesions pose a high risk of morbidity, severe pain, systemic infection, and even severe loss of bone (i.e., periapical periodontitis) and/or facial structure. This risk is further exasperated in combat situations like those seen during Operation Iraqi Freedom and Operation Enduring Freedom, which saw 20-25% deployed soldiers experienced a dental emergency, with nearly half requiring endodontic intervention. To return our warfighters to combat, and ensure their long-term effectiveness, it is vital that we better understand this disease and how to most efficiently and effectively control it. Apical periodontitis related infections are commonly populated by specific genera of bacteria such as *Actinomyces, Campylobacter, Dialister, Eubacterium, Enterococcus, Filifactor, Fusobacterium, Olsenella, Parvimonas, Peptostreptococcus, Porphyromonas, Propionibacterium, Pseudoramibacter, Streptococcus, Tannerella, Treponema,* and *Veillonella*. Naturally occurring bacteriophages have been documented for few of these genera and there is still a pertinent knowledge gap present. Additionally, apical periodontal infections are traditionally treated with antibiotics. However, the total removal of both pathogenic and nonpathogenic bacteria potentially allows for the establishment and proliferation of harmful fungal populations in these deep tissue spaces. A treatment to target the pathogenic bacteria to control oral infections long-term is needed. Phage-derived proteins have potential to be used as a treatment method for infections stemming from apical periodontitis but must be further explored and refined. Our lab currently has plaque and saliva samples obtained from patients suffering from periapical lesions for further use in metagenomic analysis of both bacterial and fungal populations. Additionally, we have partnered with wastewater treatment facilities to obtain wastewater for the discovery of bacteriophages specific to the microbial populations associated with periapical lesions. The observed phages will be recorded and further analyzed for potential use in phage-therapy for the treatment of hard-to-reach periapical lesions, while maintaining a healthy population of beneficial microbes.

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convolutional neural network (CNN) computer vision VLPs

CYCLOPS: Automated Quantification of Viral-Like Particles Using a Tunable Point-SpreadFunction and Data-Driven Blind Deconvolution

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Viruses represent the most numerous ‘‘biological entity,’ on Earth, that are the unseen driver of global biogeochemical processes, through viral lysis, selection, and genetic exchange. Globally viral abundance has been estimated to be the 10^{31}, known as the ‘Hendrix product’ which predicts that viral abundance (via viral-like particles - VLPs) is larger then the stars in the observable universe (10^{21}). Counting viruses in aquatic, soils, sediments, vertebrates, and mats have had ongoing methodological issues. Such issues include time consuming human counting by eye to enumerate viral abundance, and also lacks accurate sizing to eliminate false positives/negatives, generated by human error. To obtain better estimates of VLPs, we have developed a novel intelligent algorithm for computer vision via a convolutional neural network (CNN). This automated software CYCLOPS resolves VLPs within microscopy images using a cost-effective, user-friendly method for pennies on the dollar. Our software with modified microscopy methods provides viral abundance, viral size, and nucleic acid content abundance. We have furthermore streamlined the sample processing to better estimate viral abundances across multiple biomes including soils/rhizosphere, roots, mats, and vertebrates (e.g., fish). We can accurately size viruses from 50 - 500 nm at high resolution within a complex environmental sample in <1 minute per image. Our method also enumerates giant viruses >500 nm with a novel sample preparation that removes bacteria but keeps large viruses intact for counting. Still, challenges remain to complete enumeration for VLPs which include: 1) specific dyes for ssDNA and RNA within a dsDNA VLP mixture, and 2) fixation and preparation for membranes/lipid-containing VLPs. Our computer vision software combined with single virion sequencing with our could illuminate the unseen viral diversity present on our planet.

Klebsiella variicola | whole genome sequencing | antibiotic resistant

Complete genome of a muti-drug resistant *Klebsiella variicola* strain uncovered a novel bacteriophage isolated from wastewater treatment plant

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The *Klebsiella pneumoniae* complex consists of seven species that are related to *K. pneumoniae*, including *K. variicola*, which may colonize a variety of hosts, including environmental sources. The importance of *K. variicola*infection is obscured by imprecise detection methods, however, next generation sequencing technology has been used to correctly identify this species. Long-read sequencing is particularly helpful for the analysis of mobile genetic elements carrying antibiotic resistance genes, such as plasmids and bacteriophages. The *K. variicola* strain determined in this study was obtained from a wastewater treatment plant in the North West Province. Long reads sequencing using Oxford Nanopore Technology (ONT) was employed to profile antibiotic resistance. This strain completely assembled into a chromosome, five plasmids, and a novel 38 099 bp bacteriophage. The chromosomal genome consisted of genes *oqxAB*, *fosA5*, and *bla*-LEN that conferred resistance to phenicol/quinolone, fosfomycin, and beta-lactam, respectively. Plasmid AA035 conferred genes for resistance to metal and heat element subtypes, i.e., silver, copper, and tellurium. The phage was determined to be a temperate that consisted of a *fosB* gene and conferred resistance to fosfomycin. The temperate phage will enhance our understanding of its impact on disseminating *K. variicola* isolates and how it will impact disease development and antibiotic resistance mechanisms in wastewater treatment plants. This study highlights the need for ongoing genomic epidemiology surveillance of environmental *K. varricola* isolates as well as temperate phages.

StM171, a *Stenotrophomonas* bacteriophage with effects on antibiotic activity against biofilm formation

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Stenotrophomonas maltophilia is associated with respiratory infections and high mortality rates for immunocompromised patients. Currently, only around 43 *S. maltophilia* bacteriophages are studied, and their effects when used with antibiotics against biofilm formation are poorly studied. Bacteriophage StM171 was isolated from hospital waste water, it is a *Caudovirales* lytic bacteriophage and represents a new genus, it has a 44kbp dsDNA that encodes 59 open reading frames. StM171 has a medium host range and low burst size. It shows varying, strain-dependent and antibiotic-dependent effects on the formation of biofilms by *S. maltophilia* strains with the formation of biofilm increasing in some cases when applying StM171. *S. maltophilia* strains that developed resistance to StM171 phage showed changed susceptibility to antibiotics compared with the originally susceptible strains; most of the strains became susceptible to cephalosporin and penicillin-like antibiotics and became resistant to erythromycin and vancomycin.

Examining the effects of using bacteriophages on antibiotics and on preventing biofilm formation is an urgent question, which makes it important to further study this interaction when applying bacteriophages and antibiotics in vivo for phage therapy.

microbiome AMR plasmid phage interactome

Proximity Ligation Sequencing Reveals Dynamics Of Phage-Host And Plasmid-Host Interactome In Intestinal Microbiota Transplantation For *Clostridium Difficile* Infection

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Intestinal microbiota transplantation (IMT) has proven to be an effective investigative therapy for the treatment of chronic Clostridium difficile infections (CDI). Although this approach has shown astounding success, the analysis of response during transplantation has been complicated by the inability to associate mobile elements (plasmids and phage) with their microbial hosts without culturing. These mobile elements can transfer AMR and virulence genes from donor to host, and phages may also act to remodel the gut microbiota of people with CDI.

Proximity ligation sequencing (Hi-C) is an approach which uses crosslinks generated in vivo between the host microbial genome and the genetic material of both plasmids and phage. This crosslinked chromatin undergoes paired-end sequencing and bioinformatic processing which, in addition to recovering highly complete microbial genomes, allows for the recovery of the phage-host and plasmid-host interactome.

We applied proximity ligation sequencing (ProxiMeta™) to a time course of samples from a large cohort of patients enrolled in a clinical trial using IMT for CDI. We were able to recover a complex network of interactions between microbes and mobile elements, and uncovered the dynamics of how these networks are remodeled during the process of transplantation and recovery. We demonstrated that this method can be used to screen donors for potentially dangerous AMR and virulence genes. We also identified several phage which may play a key role in the process of microbiome remodeling. Finally, we identify phage which target AMR-containing microbes, suggesting a path towards the discovery of novel therapeutic phage.

phages machine learning genome sequencing data bioinformatics

Machine learning based phage discovery in sequencing data

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Phages are present in every living environment and play a crucial role in steering microbial population dynamics. They are important entities in our ecosystem, yet the true diversity of phages remains largely unknown. Many phages remain undiscovered, making the process of discovering novel phages challenging, time-consuming, and expensive. To expedite this process, we are working on developing an in silico tool that can accurately and rapidly recognize phage genomes in a reference- and host- independent manner.We have gathered all publicly available phage genomes and fragmented each genome into several genes-long fragments to train our machine learning model. We generated 250 genomic features to describe these fragments. Our ultimate goal is to create an easy-to-use, fast, and efficient phage prediction tool. Such a tool will enable reference-free phage discovery in sequencing data, making the process more accessible.Additionally, by extracting the most valuable features selected during training, we can gain a better understanding of the unique genome peculiarities of phages.

viral dark matter | metagenomics | next generation sequencing | transcription proteins

Discovery Of Novel Transcription Proteins From Viral Dark Matter

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With an estimated population of 10^{31} viral-like particles, viruses dominate the planet – outnumbering the entire biosphere. However, <1% have been observed and sequenced due to the constraints of host culturing. Furthermore, the lack of universal gene markers for viral communities makes them harder to study. Advances in technology, particularly metagenomic next generation sequencing, have allowed researchers to study the virome without the need for culturing hosts. Metagenomics refer to the study of all nucleic acid recovered from an environment. In a single metagenome, 60-99% of viral proteins are identified as “unknown.” These viral proteins have no sequence homology or probabilistic similarity to known proteins – making them a reservoir of unknown genetic diversity. Over the years, many scientists have used bacteriophages, or phages, to understand fundamental molecular biology; yet, how various phages “hijack” bacterial transcription activity in favor of their own genome viral remain understudied and unclear. Because transcription is essential for a phage’s life cycle, it is likely there are more viral proteins involved but have not been identified. I hypothesize that phages encode for a myriad of *unique protein structures and enzymatic abilities* involved in the synthesis of viral RNA, and with viral metagenomics and the development of a functional screen, we can elucidate the biosynthetic capabilities of phages. I will use function-based analyses to identify RNAPs or unknown proteins involved in transcription with a metagenomic library made. To accomplish this, my assays will use phenotypic complementation to screen for viral DNA that is able to rescue genetic mutations on essential genes involved in bacterial transcription. Data from this work will contribute to the field by identifying essential viral genes involved in transcription that can be used as viral markers for community studies or exploited in biotechnology.

Hologenomic evolution | viral communities | microbiome | Atlantic Salmon

Investigating Hologenomic Evolution: Exploring the Role of Viral Communities on the Microbiome of Atlantic Salmon

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In the past decade, significant advancements in high-throughput sequencing have greatly enhanced our understanding of the impact of microbial communities on various aspects, such as human health and the environment. These advancements have unveiled intricate relationships between bacteria and the host organism. Consequently, there has been a growing interest in uncovering the underlying processes that govern microbial diversity, stability, and evolution. The concept of Hologenomic evolution seeks to explain this relationship by exploring how the host organism coevolves in conjunction with microorganisms, by investigating how the host genome selects for beneficial microorganisms. However, environmental studies have demonstrated the immense impact viruses have on the microbial community ecology. In the marine environment, viruses infect 10^23 microbes every second, affecting biogeochemical cycles and shaping the microbial community composition and evolution through top-down control and impacting the genetic abilities through temperate infections. Acknowledging the influence from host-controlled environments and complex interactions with the microbiota, the role viruses play in shaping the microbiome within these environments is vastly unknown. Studies in humans have investigated patterns of viral community changes depending on disease states and viral dynamics through different life stages. In other metazoans, mainly the eukaryotic virus community has been studied, leaving significant knowledge gaps in understanding how phages interact and shape the host-associated microbiome in a host controlled environment. In this study, we aim to address this knowledge gap by using Atlantic salmon as a model organism. Atlantic salmon is a fascinating species, not only due to its importance in aquaculture or role as a keystone species in the wild, but also because salmonids have been found to have an intriguingly simple microbiome compared to terrestrial vertebrate microbiomes. In healthy and wild *Salmonella*, one genus of intracellular bacteria dominates their gastrointestinal tract, whereas sick or stressed fish have a diverse microbiome. This makes them ideal candidates to investigate how the viral community ecology is shaped in an organism that shows evidence of codivergence with its microbiome. We conducted a study to investigate the diverse viral community and its potential interactions with the host organism's microbiota. We utilized metagenomic and metaviromic datasets and employed bioinformatic tools to uncover the composition and diversity of the viruses present. Specifically, we examined distal gut samples from both farmed Norwegian Atlantic salmon with different phenotypes (sick or healthy) and wild adult Norwegian salmon. In a pilot study focused on these farmed salmon samples, we observed significant correlations between the viral population and the dysbiotic microbiome community in sick fish, while there was minimal correlation in the healthy microbiota. These findings enhance the mystery surrounding the role of the viral community in healthy and wild salmon.

Citrobacter rodentium | Phages | Virulence | Enteropathogenic Escherichia coli | Enterohaemorrhagic E. coli

The role of two temperate phage in *Citrobacter rodentium* colonization

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Citrobacter rodentium is a murine host adapted member of the attaching and effacing (A/E) family of pathogens, and is an important model in the study of the closely related diarrheal pathogens, Enteropathogenic *Escherichia coli* (EPEC) and Enterohaemorrhagic *E. coli* (EHEC). Much complexity of this model remains unknown, including the interaction between phages and the virulence of *C. rodentium*. Previous research has shown that *C. rodentium* DBS100 contains 10 prophages, while only two have been shown to be active temperate phages. This study examines 1) the *in vivo* role of phage phiNP and Shae_phiSM in the bacterium's colonization of the gut environment including 2) the *in vitro* role of these phage during bacterial competition and 3) the impact of polylysogeny on host colonization *in vitro*. We are studying the contribution of these phage to the competitiveness of their host, DBS100. Measuring maintenance of lysogeny and release of the phage during *in vivo* colonization and competition with commensal *E.coli* in the gastrointestinal (GI) tract will help us understand the role of these phage in this context. We hypothesize a competitive advantage of the lysogens compared to the nonlysogen counterpart *in vitro*, given the presence of virulence genes and the polylysogenic nature of the host. Polylysogeny, has been shown to mediate bacteria–bacteria competition, with this in mind we aim to understand how phages phiNP and Shae_phiSM contribute to the polylysogenic nature of DBS100. *In vivo*, we show that these phages enter the lytic/lysogenic cycles opposite of each other and therefore do not compete in the gut environment. We further show that Shae_phiSM is a P2-like temperate phage integrated along with a putative P4-like satellite, CRPr20. This P2-P4 interaction may be used as a model to understand one role of polylysogeny and the importance of phage interactions for the colonization of *C.rodentium* DBS100.

Klebsiella pneumoniae | prophage | depolymerase

Klebsiella pneumoniae prophages as an unexplored source of depolymerases

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The emergence of multidrug-resistant strains of *Klebsiella pneumoniae* poses a serious threat to public health. This forces us to search for alternative therapies including phage-borne capsule depolymerases. These enzymes are capable of degrading bacterial capsules, making cells accessible for antimicrobials and the immune system. Wide distribution and high diversity of prophage sequences in *K. pneumoniae* genomes could be explored for tailspikes/tail fibers with potential capsule-degrading activity.

In silico analyses of 99 *K. pneumoniae* genomes allowed us to identify ORFs encoding prophage proteins with putative depolymerase activity and with typical β-helical structure present in lytic phage depolymerases (structures predicted by AlphaFold2 software). Out of 31 ORFs selected, 5 depolymerases were successfully produced and defined in terms of substrate specificity. In addition, by site-directed mutagenesis, we identified crucial amino acids responsible for catalysis. We made assumption that substrate specificity could be predicted based on the host strain serotype, which was correct for most cases. However, two proteins showed activity against serotypes other than expected. Depolymerases 248gp38 and 914gp77 were active against K46 serotype instead of K55 and K32 serotype instead of K62, respectively.

Our results suggest that *K. pneumoniae* prophages may be a rich source of depolymerases, but the substrate specificities cannot be directly predicted based on host strain serotype, but it should be verified experimentally.

Avian pathogenic Escherichia coli | bacteriophages | colibacillosis | laying hens

Harnessing phage cocktails to safeguard laying hens from colibacillosis

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Background: Avian pathogenic *Escherichia coli* (APEC), such as O1, O2 and O78 are important serogroups for chicken health responsible for colibacillosis. With sub-therapeutic use of antibiotics being phased out in egg farms and emergence of multidrug resistant (MDR) APEC, novel approach is required to disease management.

Hypothesis: Bacteriophages, virus infecting bacteria, can be used as an alternative approach for the treatment of APEC infections in laying hens due to their efficient lytic ability against specific hosts.

Objectives: In this study, we isolated and characterized phages from hen feces and human sewage in Alberta and evaluated their potential for controlling colibacillosis in laying hens.

Methods and Results: The 7 novel APEC-infecting phages preferentially lysed APEC strains in this study and ECL21443 (O2) was most susceptible to phages (n = 5) tested. ASO78A had the broadest host range, lysing all tested strains (n = 5) except ECL20885 (O1). Phages were viable at a pH of 2.5 or 3.5-9.0 after 4 h of incubation. Comparative genomics analysis placed 6 of the 7 phages in the genus Felixounavirus (ASO1A and ASO1B), Phapecoctavirus (ASO2A), Tequatrovirus (ASO78A), Kayfunavirus (ASO2B) and Sashavirus (AVIO78A). Based on the nucleotide intergenomic similarity (<70%), phage ASO78B was unassigned a genus in the family Siphoviridae. For the bird trial, O78 targeting phage cocktail (ASO78A+ AVIO78A+ ASO78B) at multiplicity of infection (MOI >100) was introduced through oral (drinking water-DW) and intramuscular route (IM) in preventing APEC infection in experimentally challenged laying hens. Laying hens (n=35, 25 weeks) were divided in four groups 1. Medium control group (bacterial broth + phage buffer 2. DW group (phage in drinking water (4 days prior to inoculation + APEC), IM group (Phage +APEC) and APEC positive control (APEC + buffer). The mortality rate was decreased from 20% to 0% in DW and IM phage group when compared with APEC only group. Regarding body weight (BW), except for the medium control group, birds from all other experimental groups experienced weight loss over time. The group that received drinking water tended to exhibit lesser weight loss compared to the IM and APEC-only groups. The clinical score, bacterial recovery, and macroscopic lesion were greater ($P < 0.05$) in APEC followed by IM and DW group. Surprisingly, there were comparable ($P > 0.05$) moderate to severe microscopic organ lesions between the APEC only and IM groups. In contrast, all organ lesions at microscopic level were markedly attenuated from DW group. For APEC enumeration, only few APEC were recoverable from spleen of DW group, whereas greater number of bacteria were found from different organs of APEC only and IM groups. When it came to phage recovery, regardless of the phage delivery route, the spleen consistently exhibited the highest presence of phages. Furthermore, in the DW group, phages were recoverable from the liver, lungs, and heart; However, in the IM group, the phage titer decreased significantly by 7 $\log_{10}$ PFU/mL in blood samples collected 24 h after inoculation and phages were not detectable from other organs at the end of experiment. This decrease in phage effectiveness in the IM group could possibly be attributed to the rapid clearance of phages from the bloodstream and the recovery of phage mutants that displayed slightly reduced sensitivity to the phage cocktail compared to the wild type APEC.

Conclusions: Various type of endogenous phages were capable of lysing MDR APEC. Phage given in water may be valid approach for protecting laying hens form APEC infection.

Pseudomonas aeruginosa | Jamesmcgillvirus | Kochitakasuvirus | Podophages | Therapy | Tools

Untapped Wealth from Newly Characterized *Pseudomonas* Podophages: Sources of Enzybiotics, Molecular Tools, and Phage Therapy Candidates

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Pseudomonas aeruginosa is a ubiquitous and opportunistic bacteria known to inhabit diverse environments and has been frequently associated with multidrug-resistant infections in immunocompromised individuals. In nature, bacteriophages serve as the natural predators of *P. aeruginosa* and growing interest in phages has risen over the years not only for their potential to combat antimicrobial resistance but also to serve as tools for studying *P. aeruginosa* which serves a model for understanding the development of bacterial defenses against antimicrobials and phages. The study isolated and characterized 20 phages. Four were selected for sequencing and further analysis revealed that 3 phages belonged to genus Jamesmcgillvirus and 1 phage belongs to genus Kochitakasuvirus. All 4 phages contained lysis genes which could be further isolated for use in endolysin therapy while 2 were observed to exhibit lysogenic properties indicating the presence of integrases or recombinases which could be used for genome engineering. The other 2 non-lysogenic phages could become potential candidates for phage therapy, however, further characterization is required since majority of the genes in all 4 phages have unknown identities and functions which is due to the Jamesmcgillvirus and Kochitakasuvirus genera being understudied. There were also interesting observations which require further investigation like the presence of a photosystem D2 gene in one of the isolates that may indicate a much broader host range or some evolutionary consequences of phage-host interactions. Furthermore, some isolates produced multilayered plaque morphologies which are not typically observed indicating unknown phage-host interactions that is worth examining. Altogether, the findings reveal a wealth of enzymes and other genes that may be utilized for various applications.

Mycobacteria Phage

Investigating resistance to phage D29 in *Mycobacterium smegmatis*

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Mycobacterial phage D29 has a broad host range which includes pathogenic members of the genus *Mycobacterium*. Despite being well studied, the nature of the host cell receptor is not known and resistance to this phage is not well described. Here a phage-resistant mutant of *M. smegmatis* MC^2 155 was isolated by continuous exposure to low numbers of D29. Analysis of the genome sequences of the wildtype and resistant mutant identified one SNP (G insertion) in an unassigned ORF. Protein prediction tools indicated that the protein encoded by this ORF has two hydrophobic a-helices close to the N-terminus which may form a membrane spanning domain. Analysis of sequences upstream of the candidate gene identified a second ORF which encodes a homologue of the IclR (Isocitrate Lyase Regulator)-family transcriptional regulators, suggesting these two genes may form a two-component signal transduction system. Sequence analysis of this potential two gene operon identified a candidate transcriptional terminator after the second gene. Analysis of mRNA using qPCR probes targeting each of the ORFs and the intergenic region indicated these genes are expressed and do form a bicistronic operon. Interestingly, despite the known broad host range of phage D29, homologues of these two genes are not found in genomes of pathogenic mycobacteria that the phage is also known to infect. This suggests this gene system may regulate expression of the phage receptor protein in *M. smegmatis* rather than being the target for D29 infection.

Sugarlandvirus phages | Klebsiella pneumoniae | bacteriophages | antimicrobial agents

Two *Sugarlandvirus* phages reveal potential use against *Klebsiella pneumoniae* infections

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Klebsiella pneumoniae is declared by the World Health Organization as one of the top priority pathogens for creation of antibiotics. The prevalence of extended spectrum β-lactamase (ESBL)-producing and Carbapenem-resistant *K. pneumoniae* in nosocomial infections poses a grave threat to public health and calls for the development of alternative treatments. Bacteriophages or phages, known as bacteria-infecting viruses, are among the most abundant biological entities on the planet. With the low rate of antibiotic discovery in recent years, phages have re-emerged as potential antimicrobial agents that can be sustainably developed against multidrug resistant bacteria. In this study, 13 phages against clinical isolates of *K. pneumoniae* (ATCC 13887, NCTC 13438, BIOTECH 1754) with different plaque morphologies were isolated from several wastewater sources. Three phages (KP7-S1.1, KP7-JS3.1, and KP7-JS8.2) that exhibited broad host range capable of lysing the three *K. pneumoniae* hosts were prioritized. These phages, along with KP7-S2.1, were chosen for long-read sequencing due to their capacity to infect at least two *K. pneumoniae* hosts. Comparative genomic analyses revealed KP7-S1.1 and KP7-JS3.1 belonged to the genus Sugarlandvirus under the class of tailed Caudoviricites. KP7-S1.1 and KP7-JS3.1 have genome sizes ranging 109,931 – 112,834 bp and contain structural, metabolic, replication, and tRNA-encoding genes with no apparent antimicrobial resistance, lysogenic and virulence genes which strongly suggests both phages are strictly lytic. A capsule depolymerase was identified among the tail proteins of KP7-S1.1 which may be used by the phage to depolymerize the capsular polysaccharide layer of *K. pneumoniae* to allow access to receptors during phage infection. Overall, KP7-S1.1 and KP7-JS3.1 are viable candidates as antimicrobial agents. Further studies, such as determining their expanded host ranges and infection dynamics, will be important before these can be fully developed and harnessed for phage therapy.

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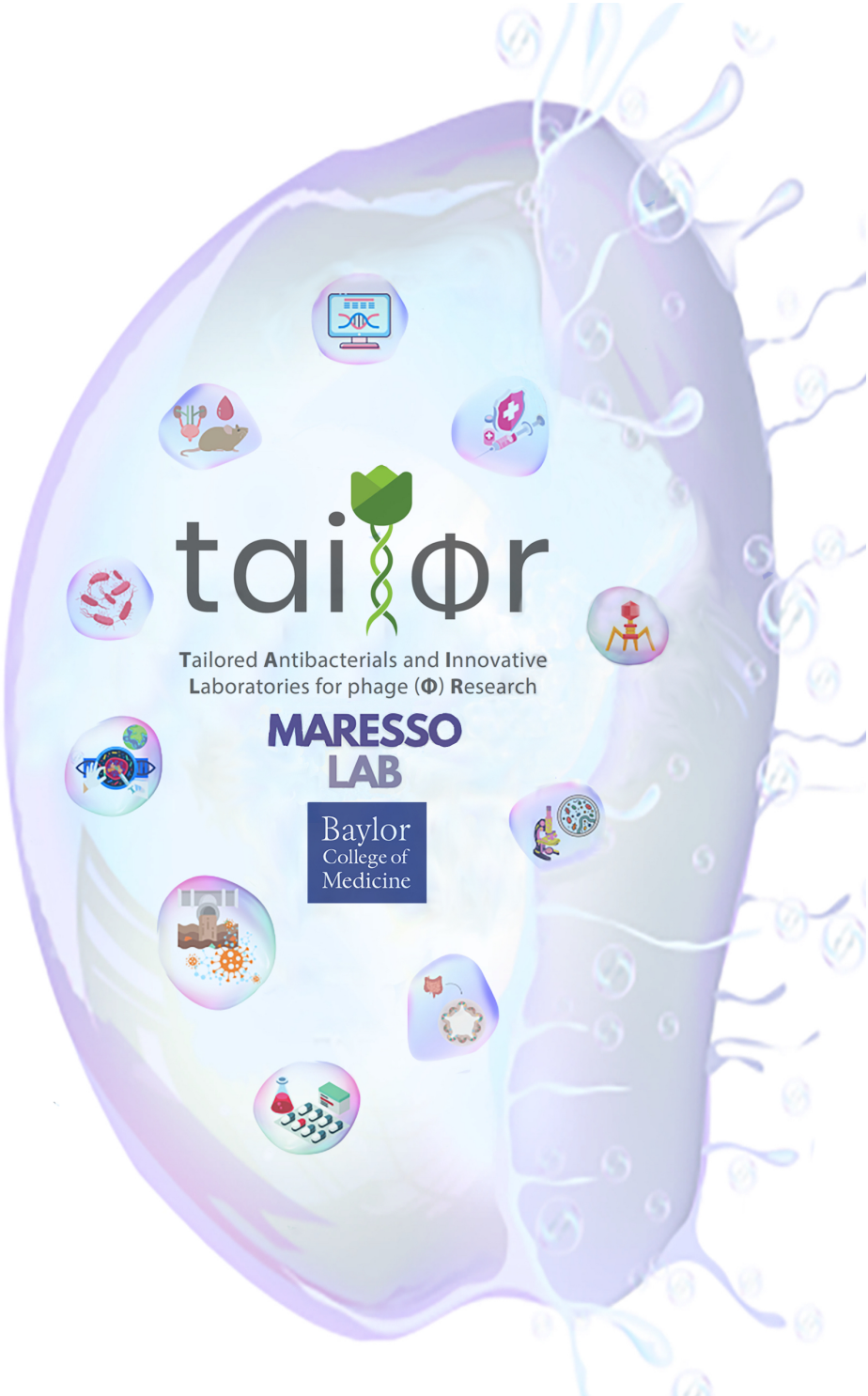
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