Old Herborn University Seminar Monograph

34. THE BIOLOGICAL EMPIRE OF THE BACTERIOPHAGE

EDITORS:

PETER J. HEIDT
JOHN BIENENSTOCK
COLIN HILL
VOLKER RUSCH

Old Herborn University

Old Herborn University Seminar Monograph 34

ISBN 3-923022-46-8
ISSN 1431-6579

Old Herborn University

Old Herborn University Foundation
Postfach 1765
D-35727 Herborn-Dill
Germany
Telephone: +49 - 2772 - 921100
Telefax: +49 - 2772 – 921101

John Bienenstock CM FRSC
1936-2022

John Bienenstock, who has been a highly valued member of the executive board of the Old Herborn University Foundation since 2017, passed away unexpectedly on July 25.

John Bienenstock was born on October 6, 1936, in Budapest, Hungary, of Jewish parents. He escaped with his parents to England in 1939, where he did grow up. He was educated at St. Paul's School in London and studied medicine at King's College and Westminster Hospital Medical School in London. He graduated with his MBBS degree in 1960.

John moved to the United States in 1964 where he was offered a position in the famous Masachusetts General Hospital. There he held a Fellowship in Rheumatology in the Lovett Memorial Group under Dr. Kurt Bloch. John trained further in mucosal immunology as a postdoctoral researcher with Dr. Tom Tomasi in Buffalo, NY. Here mucosal mast cells were first characterized by him, as was "BALT" (i.e. bronchus associated lymphoid tissue) which led to the concept of a common mucosal immune system. Both revelations have resulted in significant changes to therapeutic approaches in allergy and mucosal immunology respectively.

John was recruited to the new McMaster University Medical School in Hamilton, Ontario, Canada in 1968. He held the title of Distinguished University Professor (Medicine and Pathology) at McMaster University.

John was the Founding Director of the McMaster Brain-Body Institute at St. Joseph's Healthcare Hamilton where he studied the interactions between the nervous and immune systems. His landmark studies since 1987 on the two-way interactions and influences between the nervous and immune systems (psychoneuroimmunology) have impacted our understanding of conditions as varied as allergy and rheumatoid arthritis.
John was former Chair of Pathology and Dean and Vice-President of the Faculty of Health Sciences at McMaster University.
John received many awards and honours: he was recipient of the Order of the Red Cross in 1990, he was named a fellow of the Royal Society of Canada in 1992, and he received an Honorary MD from the Göteborg University, Sweden in 1998. In 1999 he was named Distinguished University Professor at the Mc Master University; he was named to the Order of Canada in 2002 and was inducted into the Canadian Medical Hall of Fame in 2011.
In 1996 was John invited to give a lecture on "Neuro-Endocrino Immunology"at the 10th Old Herborn University Seminar on "New Antimicrobial Strategies". Traditionally he was appointed "Honorary Professor" at the Old Herborn University and he was invited to become a member of the Science Advisory Committee. From that year on he attended all seminars accompanied by his wife Dody who, at occasions, also actively participated in the discussions.
In 2005 was John one of the moderators of the 19th Old Herborn University Seminar on "Defence Mechanisms of the Innate System; Influence of Microbes" and in 2007 was John one of the moderators of the 21st Seminar om "The Biological Significance of Gaseous Biomarkers from the Microbiota in the Alimentary Tract". In the same year, the Old Herborn University Foundation was officially registered and John became a member of the Executive Board, a function that he kept until his passing. He remained very active over the years and was one of the moderators of the 2022 Seminar on "The Biological Empire of the Bacteriophage".
We will miss him greatly and will remember him as a dear friend, a true gentleman and as a driven scientist whose ideas and discoveries will continue to have great influence on basic research as well as on the medical field.
We express our condolences to his wife Dody and his family.

Peter J. Heidt

Contents

Participating authors V

I. BACTERIOPHAGE THERAPY: CLINICAL EXPERIENCE AND CURRENT DEVELOPMENTS
(*Mzia Kutateladze*) 1

Summary 1
What are bacteriophages? 1
History of the discovery of bacteriophages and the founding of the Eliava Institute 2
Adoption of phage products in other countries 3
Decline of antibiotic research and the rise of antimicrobial resistance 3
Products of the Eliava Institute in the past 4
Main conclusions in early clinical studies 5
The Eliava Institute today 6
Main activities at the institute 6
Consortium activities – Phage therapy 6
Books on the clinical application of phages in the Eliava Library 8
Who is working on phage therapy now? 8
Web-sites and literature 9

II. GETTING PHAGES CLOSER TO CLINICAL DEPLOYMENT; URINARY TRACT INFECTIONS AS AN IMPORTANT NEED: A REVIEW
(*Martha Rebecca Jane Clokie, Thomas Erik Sicheritz-Pontén, Francesca Hodges, Anisha Thanki, Marie Noelle Vieu, Paul Robson, Andy Millard, Jill Theaker, and Melissa Haines*) 11

Summary 11
Introduction 12
The motivation for focusing on phage therapy for UTIs 14
Analysis of the systematic reviews of phage therapy 16
Assessing phage safety 18
Factors to consider for phage efficacy 20
Phages must not interfere with current available therapy 24
Lessons from animal studies 30
Clinical trial 32
Regulation 32
Conclusions and final thoughts 33
Literature 35

Contents (continued)

III. MYCOBACTERIOPHAGES: DIVERSITY, DYNAMICS, AND THERAPEUTIC POTENTIAL
(*Graham F. Hatfull*) 39

Summary 39
Introduction 39
Mycobacteriophage diversity 40
Mycobacteriophage life cycles 43
Mycobacteriophage - host dynamics 45
Therapeutic potential of bacteriophages 47
Summation 51
Acknowledgements 51
Literature 51

IV. PF BACTERIOPHAGE INHIBIT NEUTROPHIL MIGRATION IN THE LUNG
(*Medeea C. Popescu, Nina Pennetzdorfer, Aviv Hargil, and Paul L. Bollyky*) 57

Summary 57
Introduction 57
Materials and methods 58
Results 62
Discussion 65
Acknowledgements 65
Literature 66

V. TRIPARTITE INTERACTIONS BETWEEN BACTERIOPHAGES AND THEIR BACTERIAL AND MAMMALIAN HOSTS
(*Jeremy J. Barr*) 69

Summary 69
Linear relationships 69
Tripartite symbioses 70
The intra-body phageome 71
Phage uptake, delivery and activation of the mammalian system 72
Hypothetical gene potential 73
Literature 75

Contents (continued)

VI. GUT VIROME CHANGES WITH NUTRITION AND METABOLIC DISEASE INFLUENCING COGNITIVE FUNCTION
(*Anna Motger Albertí and José Manuel Fernández-Real*) 79

Summary 79
Introduction 79
Brain structure and cognitive functions 81
The gut-brain axis 82
Gut virome and metabolic diseases 83
Nutrition could impact the relationship between gut virome and cognition 84
Acknowledgements 88
Literature 89

VII. TURNING IN SILICO PHAGE INTO IN VIVO PHAGE: THE CRASSPHAGE STORY
(*Ciara A. Tobin, Andrey N. Shkoporov, and Colin Hill*) 93

Summary 93
Introduction 93
In silico discovery 94
Current classifications and general genomic features 94
In vitro isolation and biological properties 95
Ecological models 97
Conclusions 98
Literature 98

VIII. THE BIOLOGICAL EMPIRE OF THE BACTERIOPHAGE: A SUMMARY OF THE SEMINAR AND DISCUSSIONS AT THE 34TH OLD HERBORN UNIVERSITY SEMINAR
(*Colin Hill*) 101

Introduction 101
Phage therapy 101
Phage-host interactions 103
Phage genomics 104
Discussion 105

Participating Authors

Jeremy Barr, Ph.D., Head, Bacteriophage Biology Research Group, 25 Rainforest Walk, School of Biological Sciences, Monash University, Clayton Campus, Clayton VIC 3800, Australia.
(E-mail: jeremy.barr@monash.edu).

Paul L. Bollyky, M.D., Ph.D., Division of Infectious Diseases, Stanford University Medical Center, 241 Beckman Building, 300 Paseur Drive, Stanford, CA 94305, USA.
(E-mail: pbollyky@stanford.edu).

Prof. Dr. Martha Clokie, Department of Genetics and Genome Biology, College of Life Sciences, University of Leicester, University Road, Leicester LE1 7RH, UK.
(E-mail: mrjc1@leicester.ac.uk).

Prof. José-Manuel Fernández-Real, M.D., Ph.D., Department of Medical Sciences, Faculty of Medicine, Girona University, Carrer Emili Grahit 77, 17003 Girona, Spain.
(E-mail: jmfreal@idibgi.org).

Prof. Graham Hatfull, Ph.D., Department of Biological Sciences, University of Pittsburgh, 304 Clapp Hall, 4249 5th Avenue, Pittsburgh PA 15260, USA.
(E-mail: gfh@pitt.edu).

Prof. Colin Hill, Ph.D., APC Microbiome and School of Microbiology, Biosciences Building, University College Cork, College Road, Cork T12 K8AF, Ireland.
(E-mail: c.hill@ucc.ie).

Dr. Mzia Kutateladze, Director, G. Eliava Institute of Bacteriophage, Microbiology and Virology, 3 Levan Gotua Street, 0160 Tblisi, Georgia.
(E-mail: kutateladze@pha.ge).

BACTERIOPHAGE THERAPY: CLINICAL EXPERIENCE AND CURRENT DEVELOPMENTS

MZIA KUTATELADZE

G. Eliava Institute of Bacteriophage, Microbiology and Virology,
Tbilisi, Georgia

SUMMARY

The Eliava Institute is a Tbilisi, Georgia based, research organization that has been practicing phage therapy for nearly 100 years, beginning shortly after the initial discovery of bacteriophages. While phage therapy waned in the West during the antibiotic halcyon of the 1950s through perhaps 2000, the Eliava Institute continued to hone its experience in treating bacterial infections with phages. Today, it has one of the largest collections of therapeutic phages in the world. And, without question, the Eliava Institute has the broadest and longest experience of any institute in the world in employing phage therapy. The Institute's spinoff "Eliava Phage Therapy Center" is regularly and successfully treating domestic and international patients who have acute or chronic antibiotic resistant infections.

This review article discusses the discovery of the biological entity of bacteriophages, and their adoption and evolution as a principal thrust at what became the Eliava Institute. The article also follows the institute through the 20th century and shares its present structure and activities.

Research into new antibiotics has fallen precipitously in recent decades, while the rise of antimicrobial resistance (AMR) has skyrocketed globally. In a world where antibiotic resistance now threatens to kill 10 million people a year by 2050, it is highly likely that phage therapy will become increasingly important and widespread in combatting antibiotic resistant "superbugs" in the coming decades. The Eliava Institute is proud to leading the way in helping other countries rediscover the importance of phage therapy.

WHAT ARE BACTERIOPHAGES?

Bacteriophages – phages, for short – are the most abundant and ubiquitous organisms on our planet. Bacteriophages are bacterial viruses, meaning that they target and kill only bacteria. A specific bacteriophage seeks out host bacteria to which it is active, attaching itself to the exterior of the bacterial cell wall, and injecting its DNA into the bacterium. The phage's DNA then highjacks the cell's reproduction mechanism and reprograms it to produce bacteriophage particles (a process similar to many other viruses). During this active infection process and after the phage has sufficiently multiplied and assembled phage progeny within the cell, enzymes are released by the phages which lyse the outer wall of the bacteria, killing the bacteria and releasing new bacteriophage into the environment to find and attack other bacteria of that type.

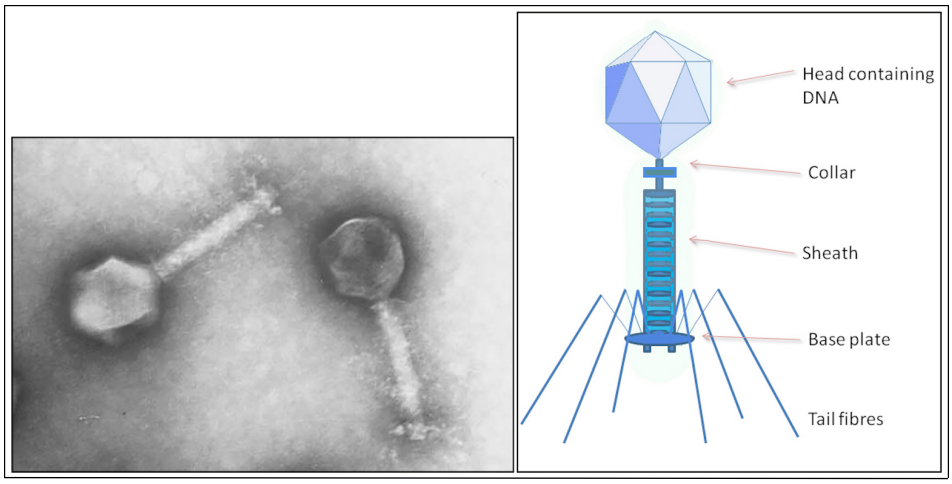


Figure 1: Electron microscope picture of a bacteriophage (left) and phage diagram (right).

Since phages multiply geometrically, a bacterial concentration (i.e., an infection) can be decimated literally within hours.

Above is described the action of "lytic" phages. Actually, phages follow two different primary life cycles. One is lytic (known as virulent) and the other is lysogenic (temperate). Mostly lytic phages are applied clinically in phage therapy.

HISTORY OF THE DISCOVERY OF BACTERIOPHAGES AND THE FOUNDING OF THE ELIAVA INSTITUTE

Bacteriophages were discovered independently by Frederick W. Twort in Great Britain (1915) and Félix d'Hérelle in France (1917). The French Canadian d'Hérelle assigned the term "bacteriophage" to his discovery, describing the viruses' bactericidal properties as a "bacteria eater". In the first human therapy, d'Hérelle treated a young boy with dysentery, drinking his concoction first to show the child's parents it was safe.

D'Hérelle soon began working with Dr. Giorgi Eliava, a young microbiologist and doctor from the Soviet Republic of Georgia, director of the Institute of Bacteriology in Tbilisi (Figure 2). These two scientists decided to create a World-center of phage research and application, and the Institute of Bacteriophages, Microbiology and Virology (IBMV) was founded in 1923. In 1989 the Institute was given the eponymic name of its founder, G. Eliava. d'Hérelle's fascinating life became the somewhat fictionalized subject of the American author, Sinclair Lewis in the novel, "Arrowsmith".

Eliava was executed during the Stalin purges of the late 1930s and d'Hérelle never returned to Georgia. But the institute thrived, particularly during World War II when phages were produced *en masse* for Soviet soldiers to treat and protect mainly from intestinal infections resulting from poor sanitary conditions and contaminated water sources.

Eliava scientists produced numerous scientific papers on phages, ranging from typology to therapy.

Figure 2: Giorgi Eliava and Felix d'Hérelle

ADOPTION OF PHAGE PRODUCTS IN OTHER COUNTRIES

In the late 1920s and throughout the 1930s, bacteriophage research and therapy were conducted in many countries – including by the U.S. Big Pharma company Eli Lilly. Numerous issues, not the least of which was the discovery of antibiotics, led to the near abandonment of interest in phages in the Western countries after World War II. But research and the prophylactic and therapeutic use of bacteriophages continued unabated in the Soviet Union and Eastern Europe, led by the Eliava Institute. Though dramatically affected by the collapse of the Soviet Union, the Eliava Institute has rebounded from the difficult decade of the 1990s and still remains today a leading organization in bacteriophage research and in the production and practical application of phage preparations.

DECLINE OF ANTIBIOTIC RESEARCH AND THE RISE OF ANTIMICROBIAL RESISTANCE

For one fleeting moment in the 1960s, some believed that the world had conquered bacterial infections through the liberal application of antibiotics. The decline in research on new antibiotics due in large part to the excessive time and cost of getting a new drug approved, coupled with the overuse of antibiotics in human treatment and particularly as unnecessary growth stimulants in livestock and poultry, facilitated the evolution of antibiotic resistant bacteria. This has created an ever-worsening situation of antimicrobial resistance (AMR). Estimates are that AMR infections will kill 10 million people a year by 2050.

In response to AMR, a growing number of institutes, companies, and government agencies are once again investigating bacteriophages as a treatment for antibiotic resistant

Figure 3: Vials of Eliava products from the 1930-40s

bacteria, and once again following the Eliava's lead in this quest. U.S. research dollars grew following the U.S. Presidential Directive against AMR (September 2014) and the United Nations resolution on AMR (September 2016). However, the U.S. Food and Drug Administration has not approved bacteriophages for treatment since rigorous investigation of safety and efficacy through clinical trials has not been conducted. In Georgia, Russia, the Former Soviet Union, and parts of Europe (Poland), phages were and continue to be a normal part of the health care system's treatment of bacterial infections. But even these countries are now encountering requirements to participate in clinical trials to demonstrate scientifically the safety, efficacy, and manufacturability of their products, and to adopt good manufacturing production (GMP) guidelines, and to seek regulatory approval for their products.

PRODUCTS OF THE ELIAVA INSTITUTE IN THE PAST

During its heyday in the 1960s through 1980s, the Eliava Institute was producing 200 metric tons a month of biological preparations (vaccines, sera, diagnosticums, and bacteriophages)! Bacteriophages for treatment and prophylaxis of bacterial infections included:

- Mono-phages (staphylococcal, streptococcal, *E. coli*, *Pseudomonas*, dysenterial, typhoid)
- Poly-phages (Pyo-bacteriophages, Intesti-bacteriophages)
- Bacteriophages for diagnostic purposes (indicatory phages).

The Eliava Institute also produced sera for treatment and identification (diphtheria, tetanus, gangrene, scarlet fever, meningococcus, *Salmonella* and *Shigella*), and various vaccines (antirabies, anthrax, *Brucella*, smallpox, intestinal)

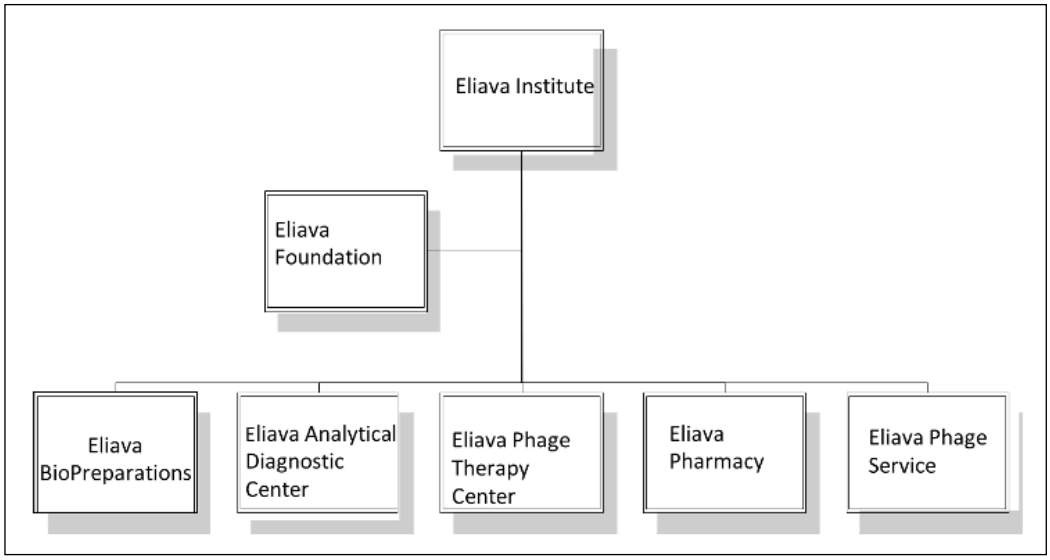


Figure 4: Organization of the Eliava Institute and Foundation

MAIN CONCLUSIONS IN EARLY CLINICAL STUDIES

Treatments in the 1930s were adopted for gastro-intestinal infections (dysentery, sub-toxic/toxic dyspepsia, colitis, Typhoid fever, Shigella, Salmonella, *E. coli* and others). While clinical trials, *per se* (double blind, placebo-controlled) were not approved by the Soviet Government, the Eliava scientists recorded observations from the institute's early years of phage treatments which can be summarized here:

- Success outcome of phage therapy is up to 95%
- Rapid improvement and cure within 3-5-7 days
- Positive cosmetic effect (no scars left)
- No relapsed cases
- No side effects
- Minimization of the mortality rate among children
- Due to prophylactic phage use, the number of actually registered disease cases in comparison with expected rates is reduced 3-6 times
- Mild disease cases in a "phaged" group
- Effect similar to vaccination
- Reduced hospital days.

Of course, these are generalizations and each assertion can be the subject of much research and discussion. But these observations continue to be the experience of Eliava phage therapy physicians even today, all indicating that the safety and efficacy of human phage treatment has been long demonstrated. The oversights in scientific method during the Soviet era that logically led to the questionable statistical significance of early work should not erase the strong empirical evidence of the positive results of human phage therapy.

THE ELIAVA INSTITUTE TODAY

To better conduct basic science in its role as a State Institution, but also to pursue commercial opportunities as a non-State entity, the Eliava institute organized itself based on a model for sustainable development. The institute remains subordinate to the Georgian Ministry of Science and Education. But, the non-for-profit Eliava Foundation is a founder of several spin-off companies. These include a bio-preparations and phage production operation, a diagnostic center, an out-patient therapy center, a pharmacy, and a catch-all entity for other services (Figure 4). Staff from the institute may work for these commercial companies, as well.

MAIN ACTIVITIES AT THE INSTITUTE

The main directions of the Eliava Institute are to:

- Conduct research on bacterial strains (including antibiotic-resistant strains), which are the causative agents of various bacterial infections
- Identify and prepare phage-based remedies against these bacterial infections.

Over the decades of its existence, the Eliava Institute has amassed a collection of more than 200 bacterial species, over 13,000 bacterial strains, over 950 individual isolates, and over 1,800 phage lysates. The principal uses of the institute's phages are for:

- Human therapy
- Animal protection (infections in poultry, cow mastitis, aquaculture)
- Plant protection (bacterial blight in cotton and rice, grape's diseases, potatoes' disease etc.)

It also has phages for protection against Agents of Biological Origin (ABO) known also as Especially Dangerous Pathogens (including *B. anthracis*, *Brucella*, *Y. pestis*, and *F. tularensis*).

CONSORTIUM ACTIVITIES – PHAGE THERAPY

The Eliava Institute scientists conduct phage research, maintain the phage and bacteria collections, and provide custom phage treatments on request. As mentioned above, the Eliava Foundation consists of several spin-off entities. These are a diagnostic center, a phage production facility, a pharmacy, a phage therapy center, and a company that can pursue other opportunities, such as bacterial decontamination. The diagnostic center and pharmacy are not specific only to phage-related activities and provide broader services.

For the present paper the most relevant consortium spin-off company is the Eliava Phage Therapy Center (EPTC). The EPTC is an outpatient clinic associated with the Eliava Institute under the Eliava Foundation structure that provides clinical services to patients with acute and chronically progressed illnesses. From 2015 through 2021 more than 1,500 foreign patients from 66 different countries have come to the EPTC for phage treatment. The number of foreign patients doubled in 2016 and tripled in 2018. Documentaries, publications and special programs made by news outlets from several countries including France, Germany, the Netherlands, Italy, Japan, Norway, the U.K.,

Table 1: Frequently Encountered Infections at the EPTC

Urologic and Gynecologic Diseases	**Prostatitis**
	Urethritis
	Vaginitis
	Cystitis, other inflammatory diseases of the urinary tract
Surgical infections	**Chronic wounds**
	Diabetic foot ulcers
	Prosthetic associated infections
Internal Medicine, ENT and Pediatrics	**Gastrointestinal tract diseases: antibiotic associated diarrhea, irritable bowel syndrome, small intestine bacterial overgrowth syndrome and infectious diarrhea**
	Respiratory system diseases: sinusitis, otitis, tonsillitis, bronchitis, bronchiectasis, pneumonia
	Cystic fibrosis
	Skin and soft tissue diseases
Frequent Bacterial Pathogens	***Staphylococcus, E.coli, Enterococcus, Pseudomonas, Proteus, Enterobacter, Klebsiella,* MDR bacteria, nosocomial infections**

the U.S. have broadened awareness of the institute. The institute's own publications also have spread awareness of phage treatment (several are included in the references.)

EPHC patients suffer from infections of different organs and systems, including surgical infections, post-operational infectious complications, prosthetic-associated fistulas, infections developed after traumatic injuries, skin and soft tissue infections, chronic osteomyelitis with the sinus openings in the soft tissue and patients with Type II diabetes (diabetic foot ulcers), etc. Generally, patients with the mentioned infections are referred to the EPTC after multiple, lengthy and ineffective treatments with antibiotics, causing the patient financial toll and the loss of quality of life.

The EPTC areas of expertise include:

- Paediatrics
- Internal medicine
- Ear, nose and throat
- Gynaecology
- Urology
- Pulmonology
- Dermatology
- Endocrinology
- Epidemiology
- Surgery

A number of the infections treated at the EPTC is shown in Table 1.

The EPTC administers phages by various methods and routes, such as oral intake, instillations into the sinus opening or fistula, wound irrigation, and by application of phage ointments on a wound or pustule. Approval for intravenous application – which was practiced during the Soviet period – is pending.

Figure 5: EPTC physicians and staff

Phage treatment is scientifically administered but also it is an artform, requiring physician decisions regarding dosing, duration of treatment, probable expected outcomes, and other factors that the practitioners have experience with.

BOOKS ON THE CLINICAL APPLICATION OF PHAGES IN THE ELIAVA LIBRARY

The Eliava Institute has a unique library with numerous books, journals, monographs, conference proceedings and dissertations and theses pertaining to biology and bacteriophages specifically. Recognizing the importance of the collection, a British donor supported the compilation and translation of some of the documents. About 5,000 items have been analysed. The institute has published two partial reviews of its holdings, including abstracts organized by topic area. One of these monographs covers the practical application of bacteriophages in medicine, veterinary sciences, and environmental research (based on old documents and publications).

WHO IS WORKING ON PHAGE THERAPY NOW?

A visceral – though irrational if phage activity is understood – public response to the use of phage treatment might be: "You are going to treat my bacterial infection with a virus? No way!" This is irrational because a bacteriophage can only attack specific bacteria, nothing else. Phages are programmed only to attach to the receptors specific to those bacteria.

This appreciation for phage therapy is beginning to be understood by clini-

cians and patients alike. In particular, when all other treatments have failed and amputation or possibly death looms, patients, doctors, and regulatory authorities all have accepted the use of phages even though general use in a country is not approved. In the U.S. the FDA approves these so-called "compassionate use" exemptions.

Clinical trials on many fronts are proceeding. Over sixty clinical trials are underway or planned from countries including the U.S., France, Poland, Belgium, Israel, Denmark, and the U.K. A wide number of infections are being evaluated, such as Urinary tract, Primary Immune Deficiency, Prosthetic Joint Infections, Cystic Fibrosis, Osteomyelitis, Shigellosis, Mild Gastrointestinal symptoms, Wound infections, Diabetic Foot infections, Staph infections, Pseudomonas infections, Enterocolitis, Venous leg ulcers, Crohn's Disease, and Acne. Some of these may include synthetic phages created or modified by new gene-splicing technologies such as CRISPR-Cas9.

It is an exciting time to be involved with what may be an old technology but one which has such important ramifications for the world's future health. The Eliava is proud to be associated with progress in this field.

WEB-SITES and LITERATURE

G. Eliava Institute of Bacteriophages, Microbiology and Virology website: http://www.eliava-institute.org/, and https://pha.ge/

BBC TV: "Could viruses called bacteriophages be the answer to the antibiotic crisis?": http://www.bbc.co.uk/programmes/articles/yGJ6MtYrP2S0gjssltQBS2/could-viruses-called-bacteriophages-be-the-answer-to-the-antibiotic-crisis

Norwegian TV: "Virus kan drepe multiresistente bakterier": http://www.tv2.no/v/1065610/

N. Chanishvili, A Literature Review of the Practical Application of Bacteriophage Research (Bacteriology Research Developments). 1st Edition. Nova Biomedica (2012).

Zhvania P., Hoyle, N., Nadareishvili, L., Nizharadze, D., and Kutateladze, M.: Phage therapy in a 16-year-old boy with Netherton syndrome. Front. Med. 4: 94, 1-5 (2017).

Hoyle N., Zhvaniya, P., Balarjishvili, N., Bolkvadze, D., Nadareishvili, L., Nizharadze, D., Wittmann, J., Rohde, C., and Kutateladze M.: Phage therapy against Achromobacter xylosixidans lung infection in a patient with cystic fibrosis: A case report, Res. Microbiol. 169, 540-542 (2018).

Corbellino, M., Kieffer, N., Kutateladze, M., Balarjishvili, N., Leshkasheli, L., Askilashvili, L., Tsertsvadze, G., Rimoldi, S.G., Nizharadze, D., Hoyle, N., Nadareishvili, L., Antinori, S., Pagani, C., Scorza, D.G., Romanò, A.L.L., Ardizzone, S., Danelli, P., Gismondo, M.R., Galli, M., Nolrdmann, P., and Poirel, L.: Eradication of a Multidrug-Resistant, Carbapenemase-Producing Klebsiella pneumoniae Isolate Following Oral and Intra-rectal Therapy With a Custom Made, Lytic Bacteriophage Preparation. Clin. Infect. Dis. 70, 1998-2001 (2020).

Nadareishvili, L., Hoyle, N., Nakaidze, N., Nijharadze, D., Kutateladze, M., Balarjishvili, N., Kutter, E., and Pruidze, N.: Bacteriophages Therapy as a Potential Management Option for Surgical Wound Infections. Phage 1, 158-165 (2020).

Poirel L., Nordmann, P., de la Rosa, J.M, Kutateladze, M., Gatermann, S., and Corbellino, M.: A phage-based decolonization stratedy against pan-resistant enteroacterial strains. Lancet Infect. Dis. 20, 525-526 (2020).

Chanishvili, N., Nadareishvili., L, Zaldastanishvili, E., Balarjishvili, N., and

Kutateladze, M.: Application of Bacteriophages in Human Therapy: Recent Advances at the George Eliava Instatute. In: Bacterial Viruses: Exploitation for Biocontrol and Therapeutics (Eds.: Coffey, A. and Buttimer, C.). Caister Academic Press, Poole, UK, 223-256 (2020).

Zaldastanishvili, E., Leshkasheli, L., Dadiani, M., Nadareishvili, L., Askilashvili, L., Kvatadze, N., Goderdzishvili, M., Kutateladze, M., and Balarjishvili, N.: Phage Therapy Experience at the Eliava Phage Therapy Center: Three Cases of Bacterial Persistence. Viruses 13, 1901 (2021).

Johri, A.V., Johri, P., Hoyle, N., Pipia, L., Nadareishvili, L., and Nizharadze, D.: Case Report: Chronic Bacterial Prostatitis Treated With Phage Therapy After Multiple Failed Antibiotic Treatments. Front. Pharmacol. 12, 692614 (2021).

Eskenazi, A., Lood, C., Wubbolts, J., Hites, M., Balarjishvili, N., Leshkasheli, L., Askilashvili, L., Kvachadze, L., van Noort, V., Wagemans, J., Jayankura, M., Chanishvili, N., de Boer, M., Nibbering, P., Kutateladze, M., Lavigne, R., Merabishvili, M., and Pirnay, J.-P.: Combination of pre-adapted bacteriophage therapy and antibiotics for treatment of fracture-related infection due to pandrug-resistant Klebsiella pneumoniae. Nat. Commun. 13, 302 (2022).

URINARY TRACT INFECTIONS AS AN URGENT NEED FOR THE CLINICAL DEPLOYMENT OF PHAGE THERAPY

A REVIEW

MARTHA REBECCA JANE CLOKIE[1],
THOMAS ERIK SICHERITZ-PONTÉN[2,3], FRANCESCA HODGES[4],
ANISHA THANKI[1], MARIE NOELLE VIEU [1],PAUL ROBSON[1],
ANDY MILLARD[1] JILL THEAKER[1] and MELISSA HAINES[1]

[1]Department of Genetics and Genome Biology, College of Life Sciences, University of Leicester, Leicester LE1 7RH, UK
[2]Globe Institute, University of Copenhagen, 1165 Copenhagen, Denmark
[3]Center of Excellence for Omics-Driven Computational Biodiscovery (COMBio), AIMST University, Bedong 08100, Kedah, Malaysia
[4]Innovate UK KTN, Suite 218, Business Design Centre, 52 Upper St., London N1 0QH, UK

SUMMARY

Bacteriophages (phages) have a long history of use in Eastern Europe and are poised for wider exploitation as novel antimicrobials in the context of antimicrobial resistance. Integrating phages into mainstream medicine requires an in-depth understanding of phages and of regulatory, economic and practical frameworks. Here we summarise insights from a UK perspective into therapeutic phage development and detail our progress towards being able to use phages for UTIs.

Phages are of interest as new medicines to target bacterial infections that are currently difficult to treat with the available therapies, and protect the medicine that protect us, by preventing the use of last line antibiotics. A pressing need has arisen for phage products to be able to treat urinary tract infections (UTIs) caused by *E. coli* and *Klebsiella pneumoniae*. Clinical trials data are needed to ensure the safety, efficacy and clinical benefits of phage treatment according to modern criteria, motivate interest from clinicians and investment from the pharmaceutical industry and thus widen access to phages. We therefore aim to conduct a human clinical trial in participants with recurrent UTIs.

We have established a UTI phage cocktail (combination) that we are optimising through a robust analysis of the phage genomes and phenotypes. On the genome front, we implement our graph-based framework to probe the genetic relationships between phages in the absence of a common marker. We describe here our repurposed ecological framework where we contextualise phage traits such as functionality in relevant physiological conditions. Ultimately, we hope to combine these approaches and correlate phage traits with therapeutic efficacy and more easily predict which phages should be developed as treatments.

Human trials can be informed by data from large scale animal trials and we show how our recent work on swine and poultry pathogens informs phage

dosage, *in vivo* dynamics and bacterial resistance. In parallel, because we will deliver phages directly to the bladder, we are currently using a murine model to collect the necessary safety and efficacy data needed for regulatory purposes.

INTRODUCTION

Antimicrobial resistance and phages

Antimicrobial resistance (AMR) is a major global problem with 2 million deaths attributed to an AMR infection in 2019 (*Antimicrobial Resistance Collaborators*, 2022) and 10 million deaths/year predicted to be caused by antibiotic resistant infections by 2050 (*O'Neill*, 2016). There is a critical need for new therapies, in light of this increasing AMR and a general lack of new antibiotic development.

To move from these startling statistics to the impact of AMR on individuals, we are often sent incredibly powerful letters and emails from patients with AMR resistant UTIs, who talk of their pain, difficulty and frustration caused by lack of access to effective treatments. We are grateful for the insights from patients who motivate us to try and find solutions. Phages are a disruptive technology and, in many ways, their lack of commonality with chemical antimicrobials along with concerns over problems of securing intellectual property and investment have flummoxed the pharmaceutical industry. We hope our work and that of others can one day direct patients' to a source of available phages within the NHS.

Bacteriophages (phages) are naturally occurring viruses that kill bacteria and thus can be used as an alternative to

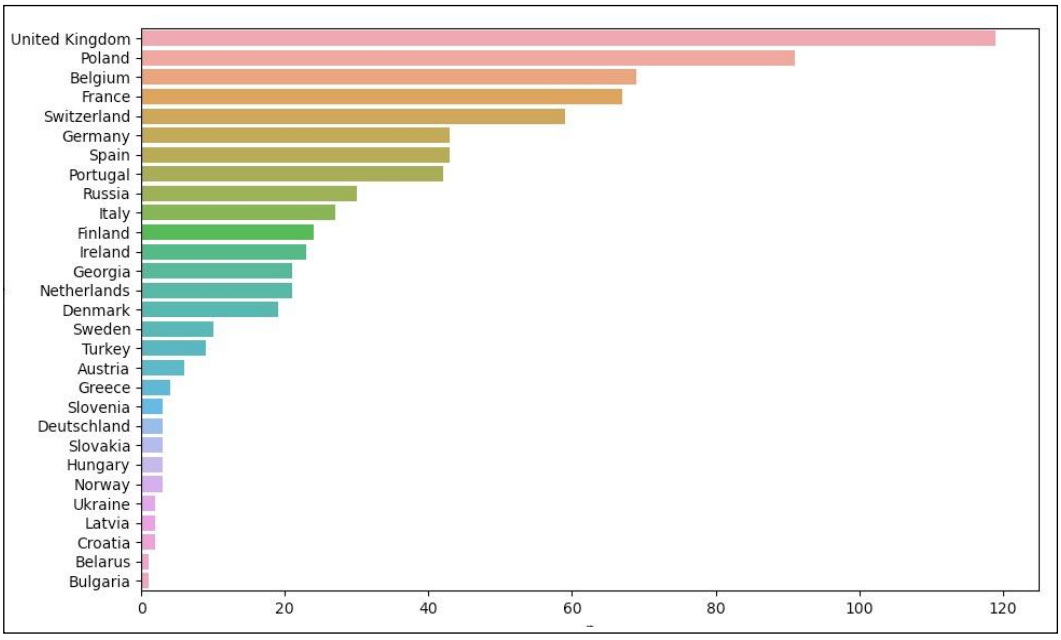


Figure 1: The number of papers on phage therapy published by country in Europe since 1941. Phage therapy papers were identified using the search terms: (PubMed entrez search for "phage therapy", or "phage cocktail", or "bacteriophage therapy", or "bacteriophage cocktail".

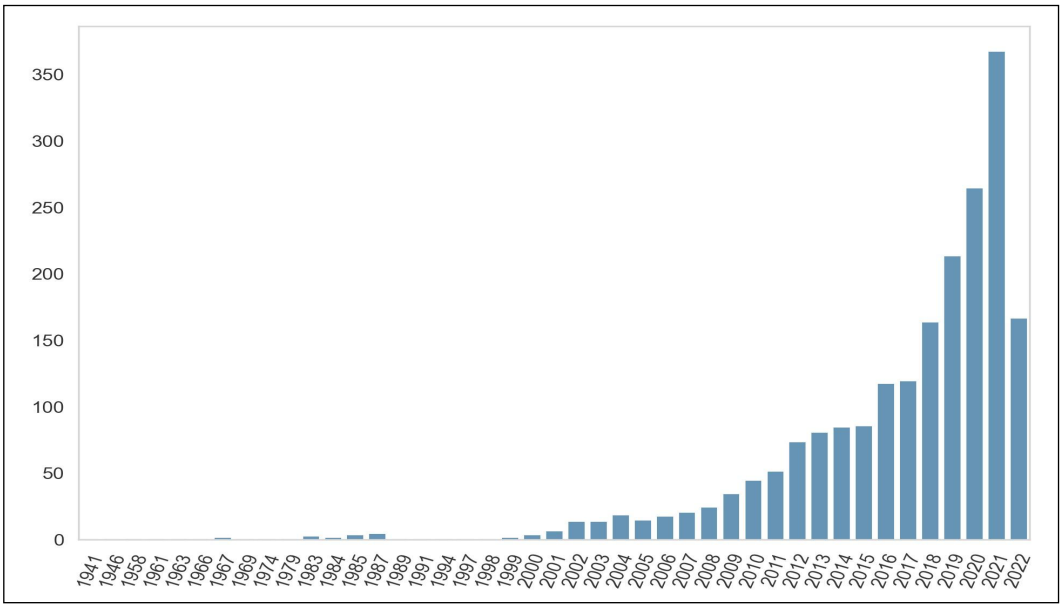


Figure 2: Number of phage papers published over time. Number of phages therapy papers published globally from 1941-2022.
Phage therapy papers were identified using the search terms: (PubMed entrez search for "phage therapy", or "phage cocktail", or "bacteriophage therapy", or "bacteriophage cocktail".

antibiotics in order to prevent or treat bacterial diseases. Although phages were discovered in 1915 and 1917 and used as antimicrobials soon after this, they were not widely used after antibiotics were discovered. Phages were seen as being problematic due to their narrow specificity towards individual bacterial species, or often a subset of strains within a species, and because of the inherent complexities associated with developing a biological entity in comparison to an antibiotic (*Gordillo* et al., 2019).The routine use of phages as antimicrobials in Georgia and Russia and for compassionate use in some European countries such as France and Poland (*Gordillo* et al., 2019) did continue (*Abedon*, et al., 2011) and currently Belgium has been increasing the amount of phage use for compassionate cases (*Djebara* et al., 2019). In the UK there are limited compassionate use studies e.g. *Dedrick* et al. (2019). This relatively small number of phage treatments largely reflects concerns regarding the safety, efficacy and consequences of using an unlicensed product, the lack of availability of phage products, and a lack of knowledge from the medical community.

Evidence for interest in the use of phages to treat bacterial diseases in Europe can be seen in Figure 1 which shows the number of papers on phage therapy published in Europe since 1941 by country. Figure 2 shows how the total number of phage papers written since 1941 has increased each year. Grant funding across the world and resources invested by biotechnology, pharmaceutical and agricultural companies have also increased.

In order for phage treatment to be accepted in the UK, it is essential to have doctors and patients' interest, awareness and understanding of how phages work and how they differ from antibiotics. The Medicines and Healthcare products Regulatory Agency

(MHRA) will also need to approve phage products.

The relative benefits of personalised and standardised phage products have been extensively reviewed elsewhere (*Pirnay* et al., 2011) and are not the subject of this paper. There is a role for personalised phage treatment in cases where emergency treatment is required (in case of multi-antibiotic resistant bacteria, or complications secondary to antibiotic use). However, there are currently no effective UK structures for phage provision. The practicability of administration in context of the English health care system needs to be assessed.

Progress towards a UK clinical trial and the scope of this review

In this review paper, we summarise the rationale for the development of clinical trials of phages developed to treat urinary tract infections (UTIs) caused by *Escherichia coli* and *Klebsiella*.

The following aspects are considered in the review below: a) burden of AMR in the context of UTIs; b) lessons learned from recent systematic reviews of phage therapy and rapid reviews of papers on phage toxicity; c) lessons learned about factors affecting phage efficacy from the on-going laboratory *in vitro*, *in situ*, *in vivo*, and bioinformatic work at University of Leicester.

A major hurdle to phage therapy is using optimally useful phages that have the correct target specificity. This requires well understood phage sets. Here we show how we have developed approaches and tools to study this. We highlight findings from our work on studying phage efficacy under different physiological settings. We provide a rationale for developing an ecological framework to identify useful phages according to their 'traits'. We also summarise how a graph-based analytical pipeline approach can provide additional information on phage relationships and be useful to probe different aspects of individual phages and inform phage cocktail composition. Ultimately we hope to be able to map key 'therapeutically' good traits within the graph-based system and the ecological framework, in order to expedite future development and use.

We then move to showcase data from our laboratory studies where we show how we have constructed phage cocktails (combinations of multiple phages) to kill *E. coli* and *Klebsiella*, and how we are building on this work to create improved cocktails with optimal physiological properties and host ranges. There is a lack of pharmacokinetic data within phage therapy on how to best deliver and formulate bacteriophages and indeed what the optimal phage dose is. We have recently carried out trials in animal settings, both in swine and poultry and present insights from these studies focusing on how they inform the design of our human clinical trial.

The regulation regarding phage products has also been the subject of much review across the globe [8] and is not a main focus here. Regulatory bodies within the UK are becoming increasingly engaged in the development of regulatory frameworks for non-traditional antimicrobials. The conclusions of the review include suggested next steps for the research groups such as ours who wish to advance phage therapy.

THE MOTIVATION FOR FOCUSING ON PHAGE THERAPY FOR UTIS

Literature studies and consultation with general practitioners, clinical microbiologists, infectious disease specialists and urologists highlighted innovative Urinary Tract Infection (UTI) treatments as a priority because of their high burden

on health care and patients, and because they can act as a driver and spreader of antimicrobial resistance.

Each year globally there are 400 million UTIs diagnosed and 236,786 UTI-related deaths (*Zeng* et al., 2022). In England UTI-related morbidity, mortality and NHS costs are high with 175,000 UTI-related hospital admissions annually at a cost of over £450 million. A third of all UTI hospital admissions have a length of stay greater than 7 days (*NHS England*, 2022).

UTIs are a major part of prescribing costs in primary care, and the most common Healthcare Associated Infection (HCAI) (*Mantle*, 2015). Furthermore, the number of deaths following *E. coli* bacteraemia has been increasing (*Abernethy* et al., 2016) (We have used the 2019-20 data to avoid confusing the story with a COVID19 confounding effect). *E. coli* bacteraemia is estimated to cost £20 million per year in England. Resistance to antibiotics increases the cost per infection by £180 - £430 depending on the resistance type (*Naylor* et al., 2019). Indeed, *E. coli* is the commonest cause of Bloodstream Infections (BSIs) and UTIs frequently lead to BSIs. In England in 2020 the number of *E. coli* BSIs was estimated at 37,823 and over a quarter of *E. coli* BSIs (29.3%) were resistant to one or more antibiotic treatment. The resistance in *E. coli* isolated from blood resistant to third-generation cephalosporins has increased significantly (*UK Health Security Agency*, 2021).

UTIs and AMR

This burden will increase further as bacteria become more resistant to antibiotics resulting in additional deaths and disability (*Cassini* et al., 2019). The key bacteria that cause both UTIs and sepsis are *E. coli* and *Klebsiella* along with Enterobacter species (*UK Health Security Agency*, 2021; *Medina* and *Castillo-Pino*, 2019). These bacteria are classified in England as 'key pathogens' because of their rapidity in developing AMR, a lack of alternative antibiotics to treat them and the fact they cause common infections such as UTIs (as well as BSIs, pneumonia and surgical wound infections).

The healthcare problems associated with *E. coli* and *Klebsiella* are compounded by antibiotic resistance, usually caused by Extended-Spectrum Beta-Lactamases (ESBL). ESBL-producing *E. coli* and *Klebsiella* are on the list of World Health Organisation 'priority pathogens' which is the highest category (*World Health Organization (WHO)*, 2017). Of major concern, both *E. coli* and *Klebsiella* are becoming more resistant to antibiotics even amongst antibiotics that are in the 'Watch' and 'Reserve' categories [18].

The inappropriate antibiotic prescribing for UTIs is a key driver of antibiotic resistance (*Pujades-Rodriguez* et al., 2019). To address this, a main target of the national AMR plan, 'Tackling antimicrobial resistance 2019 to 2024: the UK's 5-year national action plan', is to halve healthcare associated Gram-negative bloodstream infections (GNBSIs) and therefore the risk to develop sepsis. One of the priorities to achieve this target is to reduce urinary tract infections (UTIs) including catheter associated UTIs.

Therefore, as a test case to progress lytic phages towards a widespread clinical use in the UK we have focused on treating UTIs. Phage therapy is a potentially effective and safe complementary measure to the antibiotic treatment of UTI or CAUTI (Catheter Associated Urinary Tract Infections) when requiring last line antibiotics treatment. Treating UTIs and its complications could contribute to protecting antibiotics and prevent further antimicrobial resistance, which are key priorities in the UK 5

Years national AMR plan (*Pujades-Rodriguez* et al., 2019). Phages could do this by:
a) reducing the consumption of antibiotics associated with recurrent UTI, therefore antibiotic consumption for UTIs;
b) reducing the risk of recurrent UTIs becoming resistant to 'Access Antibiotics' and,
c) reducing the risk of complications of recurrent UTIs including BSIs.
Another key part of this strategy document - of key importance to our work is that 'bacteriophages' are now included as part of the new therapeutics for development.

ANALYSIS OF THE SYSTEMATIC REVIEWS OF PHAGE THERAPY

Our clinical trial work builds on a body of previous phage clinical trials and and we summarise data from this work. To synthesise all available information from previous phage therapy trials, electronic databases were systematically searched (June 2022 for all systematic reviews about phage therapy in humans) and analysed here. This identified 14 papers that had systematically reviewed clinical trials, case studies and case series of phage therapy in humans. All of these papers were published in the last five years, further demonstrating the growing interest in human phage therapy.

The following paragraphs are a preliminary analysis of these thirteen systematic reviews (*Rahimzadeh* et al., 2018; *Dąbrowska*, 2019; *El Haddad* et al., 2019; *Saperkin* et al., 2019; *Clarke* et al., 2020; *Steele* et al., 2020; *Geneviere* et al., 2021; *Kenneth*, et al., 2021; *Liu* et al., 2021; *Thomas* et al., 2021; *Aranaga* et al., 2022; *Özal* et al., 2022; *Uyttebroek* et al., 2022; *Walter* et al., 2022) that mainly identify case studies and case series of human phage therapy, and randomised control trials (RCTs). This lack of high-quality trials means that there are knowledge gaps in many aspects of phage therapy (*Saperkin* et al., 2019; *Uyttebroek* et al., 2022).
All the papers analysed conclude that phage therapy, using lytic phages, is a promising alternative antimicrobial strategy. Complex and intractable infections, due to bacterial strains resistant to available antibiotics, do respond to phage therapy (*Clarke* et al., 2020).

Phage pharmacokinetics (how the host affects the phage's absorption and distribution within the body tissues and microbiota) and pharmacodynamics (how the phage affects the host through toxicity, side effects and inhibition of bacterial growth) affect the success of phage therapy (*Dąbrowska*, 2019; *Suh* et al., 2022). Phage therapy is effective in treating bacterial infections (excluding infection caused by intracellular pathogens) provided that the following factors are taken into consideration: the use of specific phages for each bacterial strain (*Aranaga* et al., 2022), the precise characterisation of the bacteria and its concentration at the site of infection, the co-infection with other species of bacterial strains; the mode of administration (*Dąbrowska*, 2019; *Suh* et al., 2022); the phage concentration at time of administration, its dosage and frequency of administration; the patient's clinical condition; the potential development of phage resistant bacteria; the concomitant administration or not of antibiotics. When effective, phage therapy reduces bacterial concentrations and degrades biofilms thus allowing healing and improving outcomes. This occurred through the administration of either a single phage or a phage cocktail.

Systematic reviews of phage therapy

on specific infection sites have been for bone, joint and prosthetic infections, burn wounds and superficial infections; for these sites of infection, phages can be administered topically or by direct injection to the infection site. Although oral administration effectively delivers phages to the alimentary tract, it is the least effective for systemic phage penetration. The most efficient delivery was achieved by injections (intravenous - IV, intraperitoneal - IP, or intramuscular - IM) that led to effective phage dissemination within minutes, but these delivery routes are likely to generate a greater response from the immune system (*Dąbrowska*, 2019).

In most studies reviewed, phage treatment is considered to be safe because there are no adverse effects either in recent trials, case studies or case series. Most studies however do not include a deep analysis of safety and toxicity issues and indeed comprehensive and standardised reporting of potential toxicities associated with phage therapy is generally lacking in the published literature (*Liu* et al., 2021).

The heterogeneity of the studies included in the systematic reviews means that it is difficult to draw conclusions (*Walter* et al., 2022; *Suh* et al., 2022). The studies covered a wide variety of pathologies (from mild to life-threatening) and were caused by a diverse set of bacterial pathogens, infection types and locations within the body. They also differed in whether they used single phages or cocktails and in administration routes (intravenous, oral, local, or combined). Phage therapy was sometimes combined with other treatments (such as antibiotics). In some cases, phage sensitivity testing was carried out before the start of the treatment, in others it was not (empirical phage therapy with a standardised phage cocktail), or this information was lacking. For some studies, no information was provided on either the phage formulations (liquid or powder) or the concentration of the phage solutions. Outcome parameters were poorly defined and the follow-up period was short, with no information on long-term effects.

In conclusion, all of the systematic human phage therapy reviews show the potential of phage therapy but stress the need for high quality clinical trials, taking into consideration the large number of variables that can affect efficacy and safety outcomes, devising treatment guidelines, or designing clinical trials and case studies. The critical analysis of clinical information, the building of strong clinical databases, the design of consensual therapeutic guidelines, and the availability of regulatory policies are essential steps that need to be taken (*Azevedo* et al., 2022). The conduct of well-designed and sufficiently powered trials would facilitate registration and wide acceptance of phage treatments (*Saperkin* et al. 2019).

The systematic reviews show more confidence for the short-term adoption of phage therapy for topical applications, and in bio-preservation, biodecontamination and bio-sanitization (*Ssekatawa* et al., 2021). They express more confidence in phage therapy through individualised treatment with phages matched to the bacteria, but with an expectation that in the longer-term phage therapy can be used in the early stages of infection and on a larger scale, reducing the up-front use of antibiotics, helping to preserve them (*Suh* et al., 2022).

ASSESSING PHAGE SAFETY

An early step in developing a phage therapy clinical trial is to consider all potential safety issues with phage. We cover the general concerns in this area in order to show how we will address them in our work. Various mechanisms can cause drug toxicity: a) on-target (or mechanism-based) toxicity; b) immune hypersensitivity and immune response; c) off-target toxicity; d) bioactivation/covalent modification; e) idiosyncratic events (*Guengerich*, 2011).

The key phage characteristics that are critical to consider to assess the safety of phage treatment are listed below:

1) **Phage interaction with human cells.** Because phages replicate in bacterial cells, it was previously thought that they are unable to proliferate in eukaryotic cells (*Kwiatek* et al., 2020). However, some studies suggest that interactions between phages and eukaryotic cells are possible (*Podlacha* et al., 2021) and thus it is important to further understand this and from a regulatory perspective determine if they accumulate inside eukaryotic cells and the potential consequences of this.
2) **Phage target specificity and life cycle.** Phages have a narrow infection spectrum, and therefore leave the commensal microbiota unaffected. However, depending on the phage replication cycle, they may contain genes coding for factors that increase or generate pathogenicity of the bacteria for example, bacterial toxin genes (*Tiwari* et al., 2014). Thus, understanding both specificity and phage life cycles and how these impacts on the 'ecology' of an infection is critical to understanding their safety.
3) **Phage mediated immune responses.** Phages can elicit/ induce immune response either from a) the crude phage lysates; b) in response to the *in situ* 'lysates' coming from the destruction of the bacterial cell wall *in situ* (*Tsonos* et al., 2014; *Dąbrowska*, 2019). Understanding the extent of these reactions is key for successful phage deployment.
4) **Phage movement within the human body.** Phages are able to pass through bodily barriers such as organs (*Tsonos* et al., 2014; *Van Belleghem* et al., 2017).

Reviews of phage safety data highlight that the lethal impact of phage therapy is extremely rare (*van Belleghem* et al., 2017; *Liu* et al., 2021; *Aranaga* et al., 2022). Indeed, most published accounts of phage therapy report no adverse events after phage administration via oral, inhaled, or intravenous (*Tsonos* et al., 2014; *Principi* et al., 2019; *Kwiatek* et al., 2020; *Suh* et al., 2022). Adverse events reported were transient and very rarely required stopping the phage therapy. They included: fever, shortness of breath and wheezing; in another case hypotension, diarrhoea, epistaxis, oropharyngeal pain, cough, rhinalgia, and decreased blood bicarbonate (*Liu* et al., 2021; *Suh* et al., 2022).

As stated above, increased immune responses can occur, directly due to phages or due to the bacteriophage-mediated lysis from a large bacterial load that causes the release of endotoxins, resulting in cytokine release syndrome (*Van Belleghem* et al., 2017; *Kwiatek* et al., 2020; *Liu* et al., 2021; *Suh* et al., 2022).

Hypersensitivity and cytokine release syndromes can also be induced by bacterial components and toxins present in phage preparation (*Liu* et al., 2021). Phage preparations can also impact the innate and adaptive immune system directly, resulting in production of phage antibodies. The anti-bacteriophage humoral response seems to be dependent on a number of factors;

largely the route of bacteriophage administration and the dosage (*Principi* et al., 20019; *Kwiatek* et al., 2020; *Liu* et al., 2021).

Some chemical components used in the purification process of phages can have toxic effects for example intoxication from Caesium Chloride (CsCl) can result in gastrointestinal distress, hypotension, syncope, numbness, or tingling of the lips (*Liu* et al., 2021). Other biological effects include transaminases, transient septic episodes and anaphylactic related decompensation (*Liu* et al., 2021). Bacterial resistance to phages was reported in some clinical case reports (*Aranaga* et al., 2022).

Limitations of phage safety studies

Although minimal toxicity has been reported from both *in vivo* animal and human phage therapy studies, many concerns have little data associated with them and thus need to be taken into consideration. The distribution of phages within the body and their impact on tissues and physiological processes are largely unknown. This is in part because most of the studies focusing on this issue were carried out on temperate phages (*Liu* et al., 2021). Furthermore, the impact of phage therapy on the human microbiome is unclear (*Liu* et al., 2021).

Very few studies have documented the effects of the release of endotoxins or reported data on immunogenicity of phage (*Liu* et al., 2021). Similarly, data on phage preparation are under-reported in animal and human studies, including genotype information (*Liu* et al., 2021). Although phages are clearly distinct from standard API's (active pharmaceutical ingredients) very few studies have actually defined the median effective dose (ED50), lethal dose for 50% (LD50), or the therapeutic index (TI) (*Liu* et al., 2021). We discuss a dose response below in connection with our animal studies.

Another limitation is that no studies were found that assessed genetic toxicity of phages or the effects of phage therapy on pregnancy growth and development. A more general point is also that the animal trials were carried out on rodents and not large animals (e.g., pigs), this limits the generalizability to humans (*Liu* et al., 2021). Finally, there are currently no animal or human studies that present data on assessing the risk of emergence of bacterial resistance against phage (*Principi* et al., 2019).
Several recommendations for toxicity studies of phage therapy were made (*Principi* et al., 2019; *Liu* et al., 2021; *Suh* et al., 2022). We are currently addressing these in our pre-clinical work in anticipation of the clinical trial work.

1) **Factors relating to the phage formulation:**
   a) Describing the methods used to propagate and purify the phage preparations should be standard.
   b) Quantify and document the presence of bacterial components in phage preparation especially endotoxin.
   c) Ensure the removal of chemical contaminants in phage preparation.
2) **Factors relating to the characterisation of the phages used:**
   a) Include an analysis of immunogenicity of phages in both animal and human studies.
   b) Include information on the characteristics of phages used in animal studies and clinical studies, including their morphology, genetics, and protein profile, as well as the composition of the phage preparations, including the levels of bacterial contaminants and other impurities.
   c) Sequence the phage genome and ensure it does not contain genes

enabling the lysogenic lifestyle, antibiotic resistant genes (ARG), genes for phage-encoded toxins.

3) **Factors relating to data that should be collected from the patients:**
   a) Reports on the safety of phage therapy should include information on the general health of participants, adverse events, chemistry, and hematologic testing data.
   b) Information on immune responses should be evaluated prior, during, and after phage therapy.

FACTORS TO CONSIDER FOR PHAGE EFFICACY.

Developing well-characterised phage sets

Having set the scene for safety concerns we now consider aspects that relate to efficacy. To develop an effective phage therapy product requires an understanding of the phage specificity and behaviour with respect to the pathogen of interest. A knowledge of how many phage types are required to kill the most relevant circulating serotypes or micro diversity within that bacteria strain is essential. We have built a large collection of AMR *E. coli* and *Klebsiella* strains isolated from urinary samples from patients with UTIs and representing the most prominent circulating sequence and capsule types. This is supported by the UK literature for uropathogenic *E.coli* (*Doumith* et al., 2015; *Day* et al., 2019), unfortunately for *Klebsiella* the data on dominant serotypes is less well-defined (*Gorrie* et al., 2018; *Caneiras* et al., 2019).

One approach to minimise bacterial resistance towards phages and maximise target specificity within a species, is to administer a phage cocktail. To ensure that the phage cocktail is effective, phages in the cocktail should not hinder each other and preferably be synergistic. Experiments have been conducted at Leicester university to investigate the interactions of phages within a cocktail (*Haines* et al., 2020). A 'virulence index' has been used to define and quantify the phage cocktail efficacy (*Storms* et al., 2020). This method quantifies bacterial death by measuring the optical density of bacteria over time. Using this method, a score of '0' means that there is no impact on the bacteria and '1' is maximum impact with all bacteria killed. By comparing the local virulence index of individual phages, to that of phage combinations (doublets/triplets/sextuplets) positive and negative interactions between phages can be identified (*Haines* et al., 2020).

A clear example of synergy was seen between the phages UP17 & JK08 that target *E.coli*. The synergy was seen across several clinical isolates, but was particularly marked for *E. coli* KR2733 where virulence index for UP17 alone was 0.3, for JK08 alone was 0.45, but when both phages were used in combination the impact increased to 0.95.

The current study suggests that some phage adds to the efficacy of other phages while being of limited efficacy on their own. This phenomenon would not have been identified using the current phage selection methods. This highlights the need for well characterised phage sets to be generated in order to produce 'nuanced'- less obvious phage combinations.

The impact of Oxygen on phage infection

A number of the pathogens on the World Health Organisation's priority list of antibiotic resistant bacteria (*Shrivastava* et al., 2018) are facultative

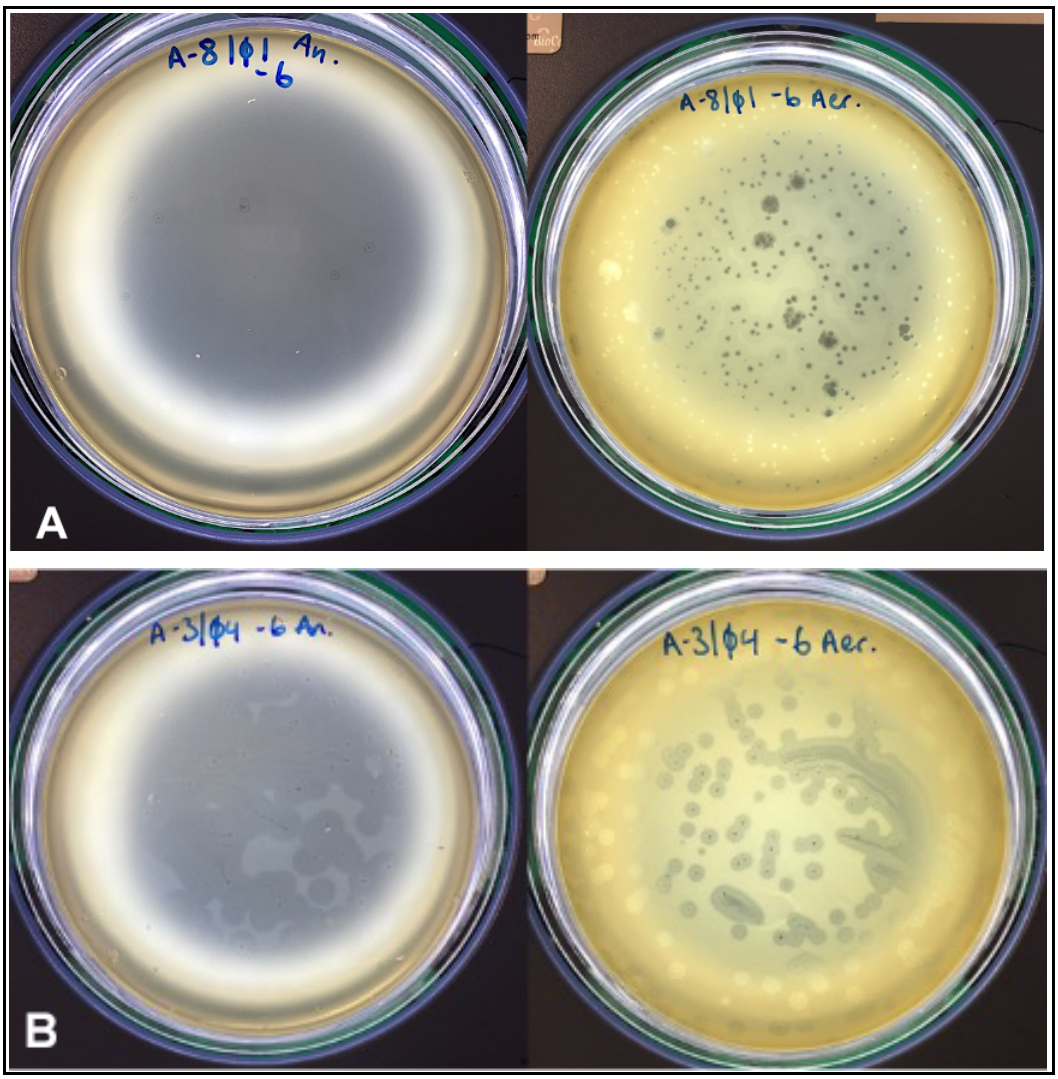


Figure 3: Specific bacteria and phage combinations respond differently to oxygen concentration.
A: The phage is unable to infect the bacterial host under standard aerobic conditions of oxygen yet when it infects the host in an anaerobic environment it grows well.
B: The opposite is true and the phage infects well under aerobic conditions

anaerobes, which means that they are able to switch between aerobic and anaerobic respiration. This allows them to survive or even thrive, in diverse environments with varying levels of oxygen available. In humans these environments include skin wounds, the urinary tract, the digestive system, and the lungs where these pathogens can cause biofilm. We showed that according to plaque morphology, some phages work infections.

It is part of phage development to assess their ability to work in conditions under which they are likely to encounter their bacterial hosts. In a bladder infection this may include the ability of phages to work under microaerophilic conditions or possibly in anaerobic conditions if the infection occurs within a better in anaerobic conditions and others in aerobic (Figure 3).

There is little in-depth knowledge of how changes in oxygen availability affects the efficiency with which a phage can infect its host. Our recent review reports on phage phenotypes under aerobic growth conditions and conditions where oxygen is limited (*Hodges* et al., 2021). Ultimately it concluded that oxygen availability can have a clear impact on phage effectiveness. This conclusion is also supported by unpublished results from our previous work which demonstrates that oxygen availability can have an effect on the burst size, latent period, adsorption rate, and efficiency of plating (EOP) measured in the characterisation of a phage (Figure 4). From this figure we can see that φA1 had a shorter latent period and faster adsorption rate under anaerobic conditions, a higher EOP ratio under aerobic conditions, and there was no significant difference measured in the burst size for this phage between aerobic and anaerobic conditions.

The review also highlights the need for standardising the measurements of phage virulence taking into consideration the bacterial environment. Measurements such as burst size, latent period, and adsorption rate are all affected by the environmental conditions under which the phage encounters its host (*Hodges* et al., 2021) and this is not usually accounted for in experimental design or virulence testing.

Data collection of phage virulence under different bacterial environments would contribute greatly to the reliability of *in vitro* phage characterisation when work is translated to *in vivo* studies and larger scale trials.

Of note, in our work we showed that different parts of a phage life cycle are 'better' or 'worse' in terms of contributing to phage reproduction, under high or low oxygen conditions. We do not currently know which of these attributes best correlates to a good therapeutic outcome, but these data suggest a lot more characterization could be done to relate these properties on a larger scale to efficacy and thus ultimately use the information to better predict which features will work well for particular infections under particular conditions.

The ability of phage to work in different pH and in biofilms

Ideally, phages should be tested in conditions that mimic the bacterial host environment. The bladder and urine are a potentially hostile, de-activating environment for phages. The pH of human urine can vary from 4.5 – 8.0, so it is important to test efficacy in this pH-range. Furthermore, a buffer could be used for the final phage cocktail for intravesical therapy within the murine model of infection and ultimately in humans. Such as salts, for example magnesium or calcium salts could also be added to improve efficacy (*Jończyk* et al., 2011).

Additional *in vitro* testing will consider the ability of phage cocktails to combat bacterial virulence factors such as biofilms (biological matrices that bacteria produce to protect themselves from the environment). Their importance in urinary tract infections has been previously shown for *E. coli* and *Klebsiella* (*Hancock* et al., 2010). Our previous work (*Haines* et al., 2020) demonstrated the efficacy of the phage cocktail against *E.coli* biofilms (11/19 isolates), but required improvement for biofilms created by *Klebsiella* (5/19 isolates). The phage cocktail has now been refined and experimental work is in progress to determine the efficacy of the improved combination.

Phage efficacy in model systems

Over the last decade we have developed several physiologically relevant models, in which to study phage efficacy (*Nale*

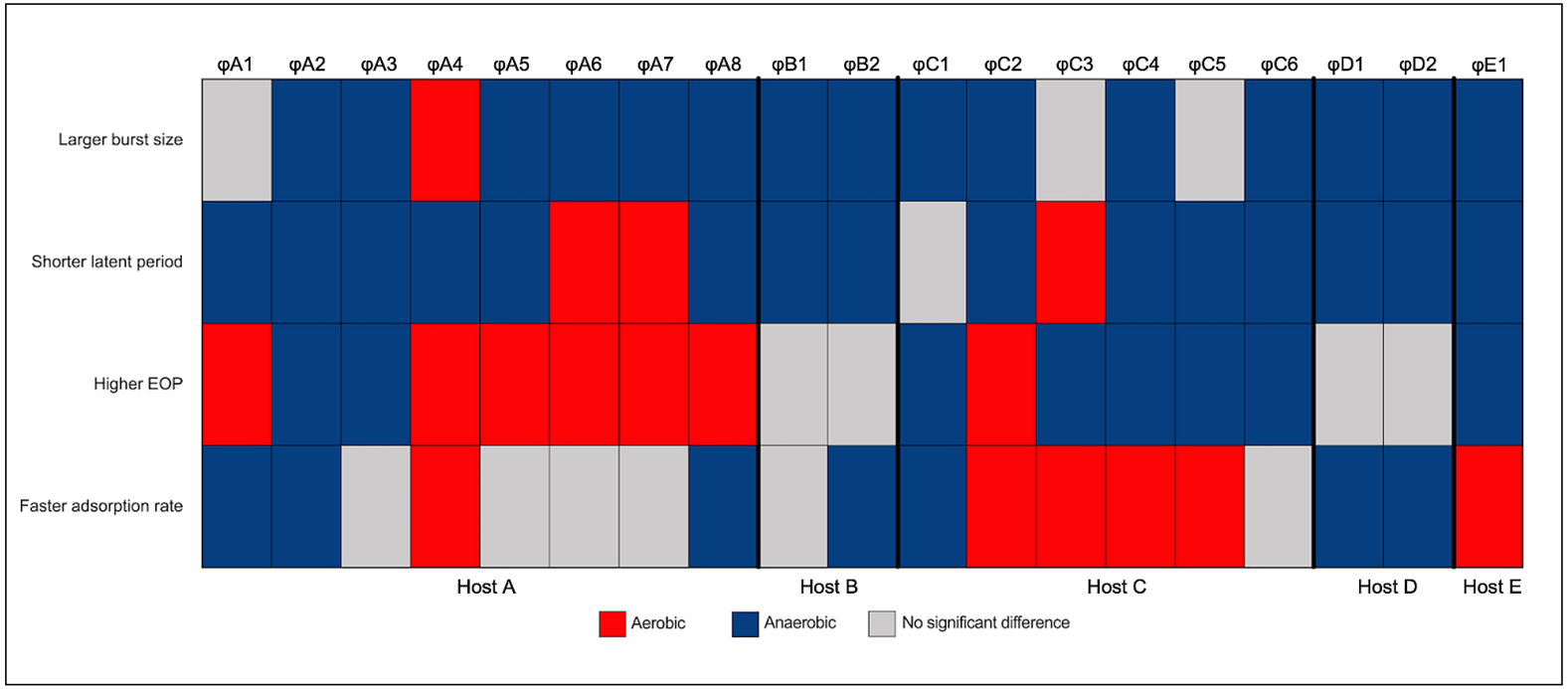


Figure 4: A heatmap (unpublished data) that indicates whether a phage infection was 'better' under aerobic or anaerobic conditions. Better is defined as potentially contributing to more phage progeny. Measurements better under aerobic conditions are labelled in red, and those better under anaerobic conditions are labelled in blue. Grey represents no significant difference.

and *Clokie*, 2021). These include a range of relevant cell lines that provide critical data on how phages interact with components of the immune system. Insect models are incredibly useful to establish dosing regimens and to look at the impact of bacteria that are resistant to phages. Artificial gut models allow the observation of how phages impact on other microbiota. For this work we have designed an artificial bladder that we will use in conjunction with catheters from patients in order to show how effective our phages are on natural UTI biofilm communities.

PHAGES MUST NOT INTERFERE WITH CURRENT AVAILABLE THERAPY

The development of phage therapy requires study of the interactions of phages with current therapy, such as antibiotics. The current work at the University of Leicester includes assessing the positive or negative interactions between common antibiotics used to treat UTIs and our phage collections. There are many examples of phage-antibiotics synergies (PAS) and an excellent review on this phenomenon (*Łusiak-Szelachowska* et al., 2022). There have been reports of instances where antibiotic resistant bacterial isolates have become sensitive after selective pressure of phage treatment.

Frameworks to recognise 'phage types' to progress therapy

Phages in their natural environments, like all viruses, have evolved to not immediately kill all of their bacterial hosts and to avoid generating resistance ie. they have a plethora of ways to ensure their survival until they can target an appropriate host. However, a subset of phages will have sets of characteristics that render them more appropriate at performing this than others. Unfortunately, it is not known in a general context which phages have optimal traits that are suitable for therapy. A major research focus within our laboratory is to do exactly that. We have repurposed an ecological framework that is based on a botanical framework (*Clokie* et al., 2020) and we are currently trying to understand what ecological features across all phage groups render particular phages effective.

The CSR concept was developed by Grimes in the 1970s to the 2000s in order to classify all UK flowering plants into a functional type with the view to better understanding individuals, communities and to be able to make predictions about what types of plant species we would expect in specific environments (*Grime*, 1979). All plants, Grimes argued, can be divided into those which are good competitors, stress tolerators or ruderals (Figure 5). Competitor plants are those that when environmental conditions are good they create a good infrastructure before reproduction, A classic example is stinging nettles. Stress tolerant plants in contrast, are highly conservative with their 'resources' such as wild thyme with tiny leaves and flowers. The final category, ruderals are good at coping with disturbance and can get to their reproductive state very quickly in order to exploit a newly formed environment. A classic ruderal is a poppy which is a symbol of war because it is one of the first plants to grow on land disturbed by trenches. Plants can also be any combination of these categories. As this scheme has stood the test of time within botany, rather than designing an entirely new set of axes from the outset we are trying to understand if this may also serve a useful purpose within phages.

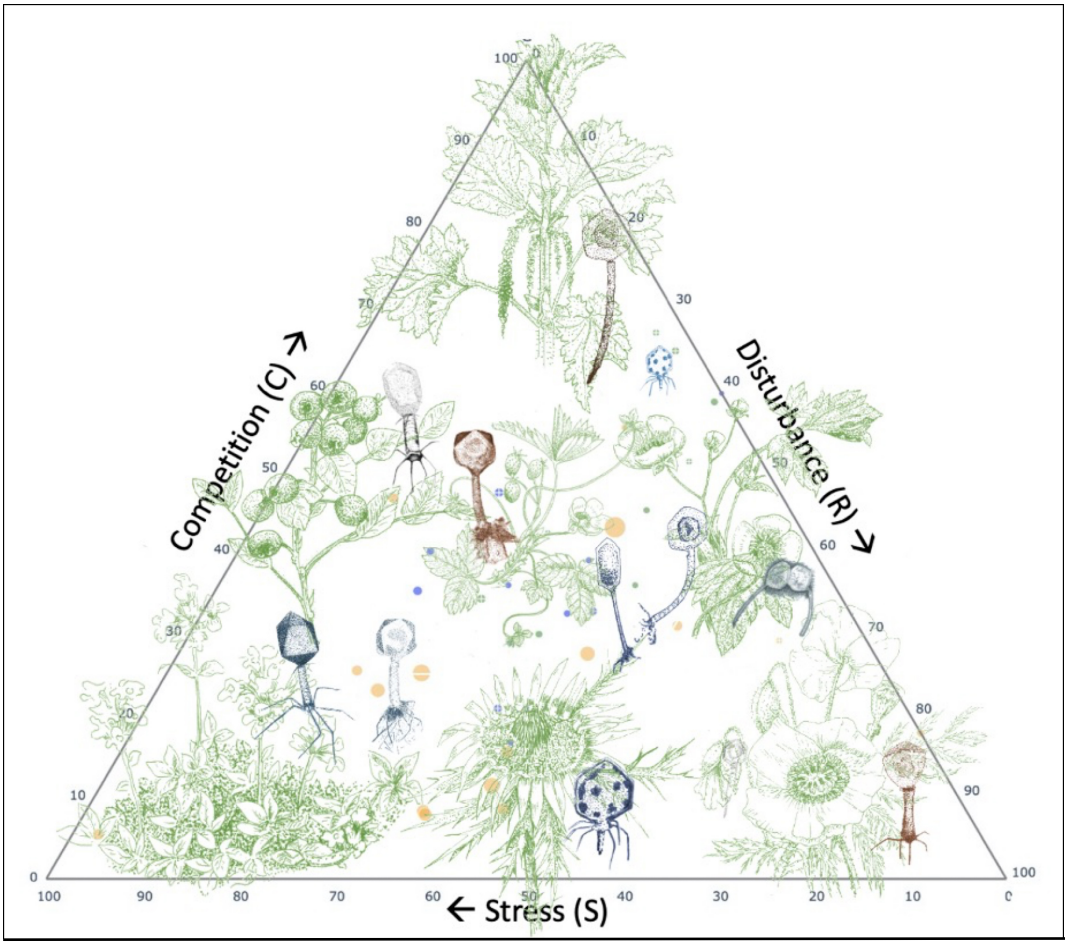


Figure 5: Plants that represent attributes according to an axis of competition (C), stress (S) or disturbance (D). The 'familiar' species we chose to depict these categories are stinging nettle (C), thyme (S) and poppy (D). CR is sea holly, CS is blueberry and CR is buttercup. Wild strawberry represents CSR. Phages have been imposed on the diagram to illustrate that they will also conform to a range of ecological strategies (*Grime*, 1979).

Phage traits are more hidden than those associated with plants, but it is likely that many traits can be predicted from phage genomes such as the genes that encode for parts of their replication and translational machinery. They are also likely to include genes that encode proteins associated with stability, or stress. As an initial proxy for this scheme, we have looked at the proportion of phage genes that are expressed at different points in the life cycle (*Clokie* et al., 2020). Based on these concepts, phages could be classified as a) competitor phage which encodes proteins that enable significant rearrangements to the bacterial cell; b) ruderal phage disturbance surviving phage) and c) stress phage which encodes proteins that facilitate the survival of the bacteria whilst replication can occur. Although we have been limited to date by the small number of phage transcriptomes that have been collected, data suggest our theory is plausible (Figure 6).

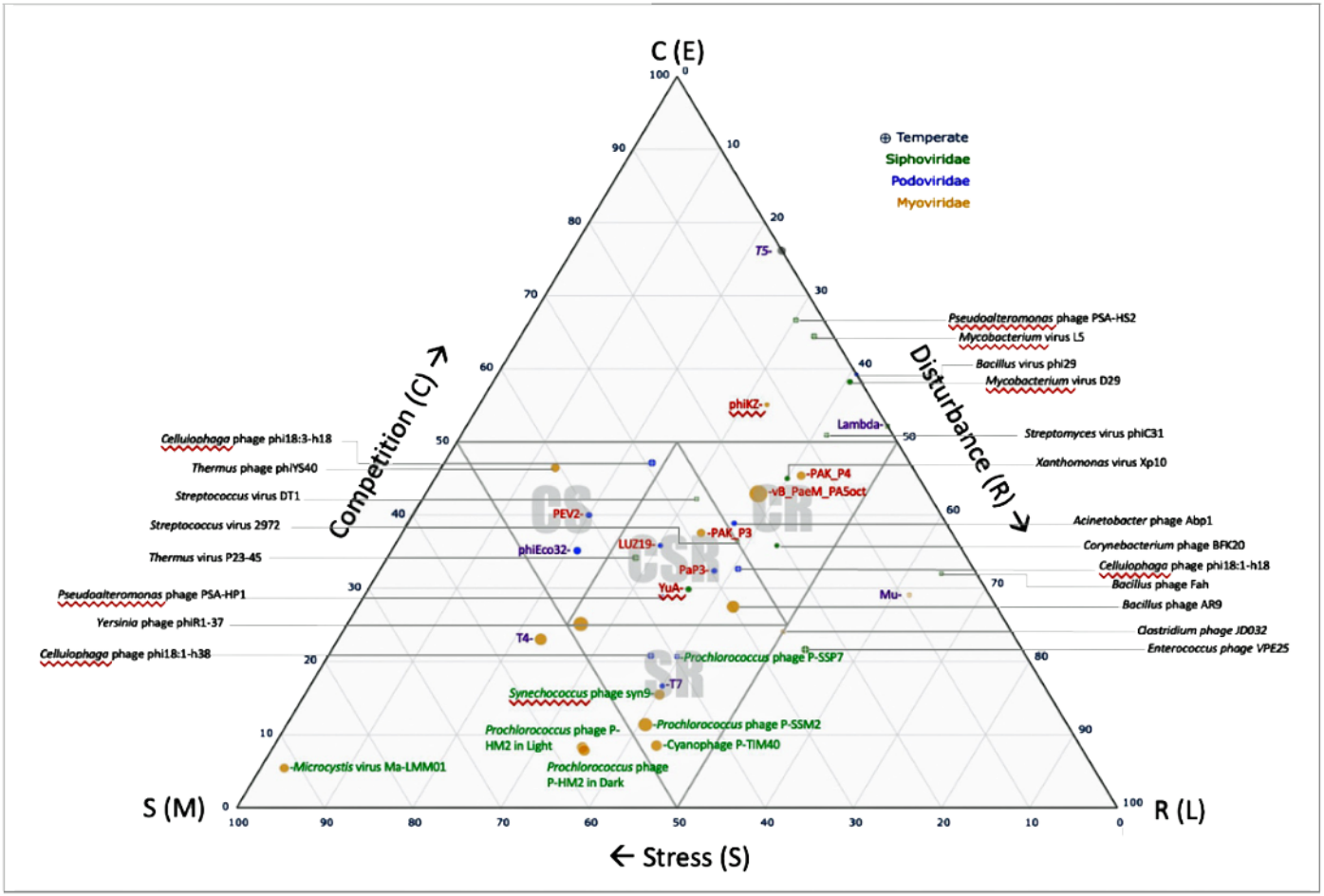


Figure 6: The position of 42 phages based on the proportions of early, middle, and late genes within the CSR triangle, which reflects their ability to tolerate competition, stress, or disturbance. The colour and size of the dots represent the phage classification into myoviruses, siphoviruses, and podoviruses and their genome size. Although the phages were all examined during their lytic cycle, those with a cross have access to the temperate cycle. Associated labels of phages that target *Pseudomonas* are shown in red, *Escherichia* in purple, and cyanobacteria in green.

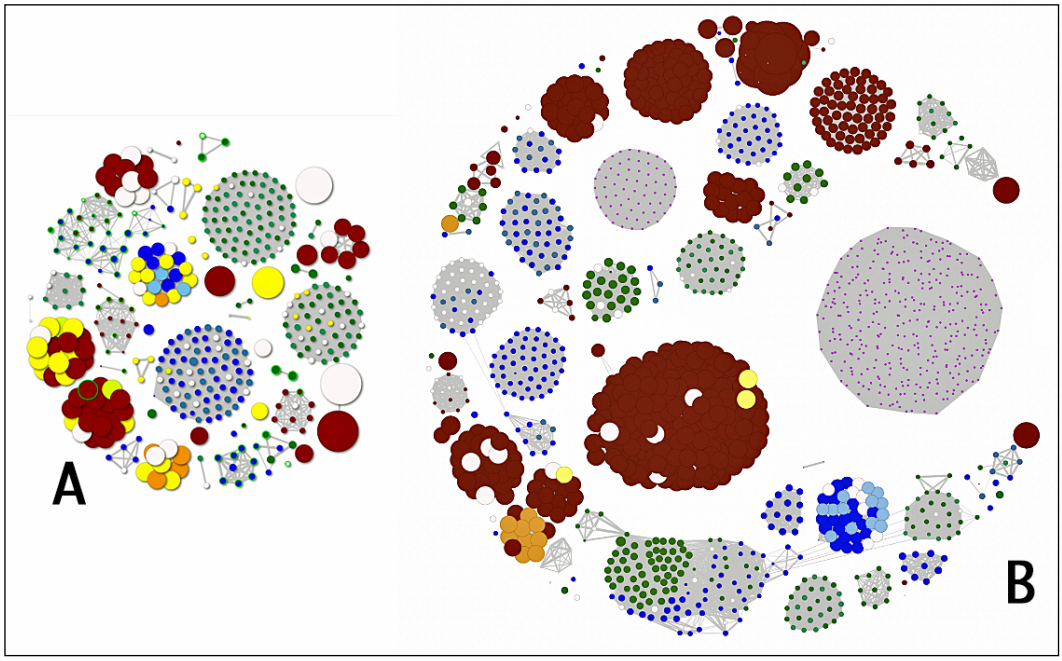


Figure 7: A schematic representing all genomes in NCBI for phages that target *Klebsiella* (A) and *E. coli* (B) respectively. The colours represent phage taxonomy.
Although recent large-scale changes to phage taxonomy have disbanded three large families, for backwards compatibility, phages from the former *Myoviridae*, *Podoviridae* and *Siphoviriade* are included alongside the recently described families. The colours represent these groupings, *Siphoviridae (cobalt blue)*, *Demerecviridae (light blue)*, *Ackermannviridae* (orange), *Microviridae* (pink) and taxonomically unclassified (white).
A) The *Klebsiella* clouds have 1270 phages within ~20 clouds.
B) The *E. coli* cloud has 1657 phages composed of ~30 clouds.
The figures are scaled so that in both cases dot size is reflective of the genome size.

Another key tool that we show data for is a graph-based method that we have developed to look at the evolutionary relationships between phage genomes. Ultimately, we hope to link these two concepts together and therefore to impose our ecological framework onto our clouds network. In this review we will showcase the clouds-based approach with respect to bacteriophages that target both *E. coli* and *Klebsiella pneumoniae*.

In the last five years, we have increasingly been working to build both experimental and computational frameworks to determine and select phages and phage combinations with maximum therapeutic suitability. To support the experimental workflow and to get a rapid overview of the phages and phage cocktails isolated and developed in our laboratory, we have developed a graph-based method to quickly characterize their genomic and evolutionary relationships (*Rangel-Pineros* et al., 2021).

To explain our graph-based method, in Figure 7A, the clouds towards 11:00 o'clock have multiple cloud connections that, from our experience, are indicative that they have a temperate lifestyle. This is consistent with the fact that they contain known prophages (*Rangel-Pineros* et al., 2021). Although there are a larger number of phages within the *E. coli* cloud (Figure 7B), it allows us to see some interesting trends. Because *E. coli* phages are comparatively well understood, we know which are temperate

and again they as expected in this figure, being within the clouds that are connected closely to each other such as those at 6:00 o'clock. It is also clear that there are clouds that contain phages with very small, and some with much larger genomes.

The phages represented by yellow dots are those that we are characterising in detail, many of which came from a collaboration with Ellie Jameson who has extensively characterised their physiological parameters and host-ranges (*Townsend* et al., 2021). We have been adding to this body of data and in general, and as expected, we notice more similar 'behaviours' in terms of physiological parameters, from phages within a cloud than between those in different clouds.

Phage traits

In addition to virulence and host-range, many other facets of phage biology are likely to be useful to inform clinical development. These are yet to be defined and referred to here as 'phage traits.' These will be the genes/proteins to render phages so effective at killing under specific conditions. They may also be traits that activate immune responses that convey less clearance from the human body - or those that can enter eukaryotic cells in order to clear infection. Conversely, it is equally important to establish which traits make phages less effective, for example what traits link to the safety questions raised above, or to the phage life phage cycle? Answering these questions would improve phage cocktail design and facilitate effective phage selection and clinical phage bank design.

One concept we are also investigating is if we can use traits/attributes from one phage taxa to inform others within the clouds described above. For example, if one cloud member carries a specific toxin gene, does the whole group have the potential ability to also do this? Similarly, if there are any temperate markers associated with the phage, or known phenotypic characteristics of transduction for example, should the whole cloud be down prioritised as therapeutic phages? Once we have a greater understanding of what traits are measurable and what they mean, we will ultimately be able to project them onto the phage clouds and also combine this with their different ecological lifestyles to help work out their therapeutic potential. For example, to be able to quickly project a phage's therapeutic suitability onto the phage clouds, we developed a machine learning based pipeline checking for presence of temperate markers, antimicrobial resistance genes, and virulence genes (*Yukgehnaish* et al., 2022).

Our efficacy studies of phage cocktails in various animal settings show that the best performing phage combinations are often those containing phages from different phage clouds with different ecological strategies. This is important within the challenge of bacterial phage resistance.

Traits that we would deem to be incredibly useful within a phage are those that allow the phage to be useful and to function in its intended location such as within a human environment. A phage ideally would also be amenable to propagation and formulation at high titre and easily separated from any potential toxins that might make infection more severe. It is also becoming increasingly clear that phages are not fully inert to human cells, some phages appear to drive an increased immune response whereas others appear to reduce the amount of cytokines and chemokines that are produced during an infection. These are likely to be traits to be taken into consideration during the selection of clinically relevant phages.

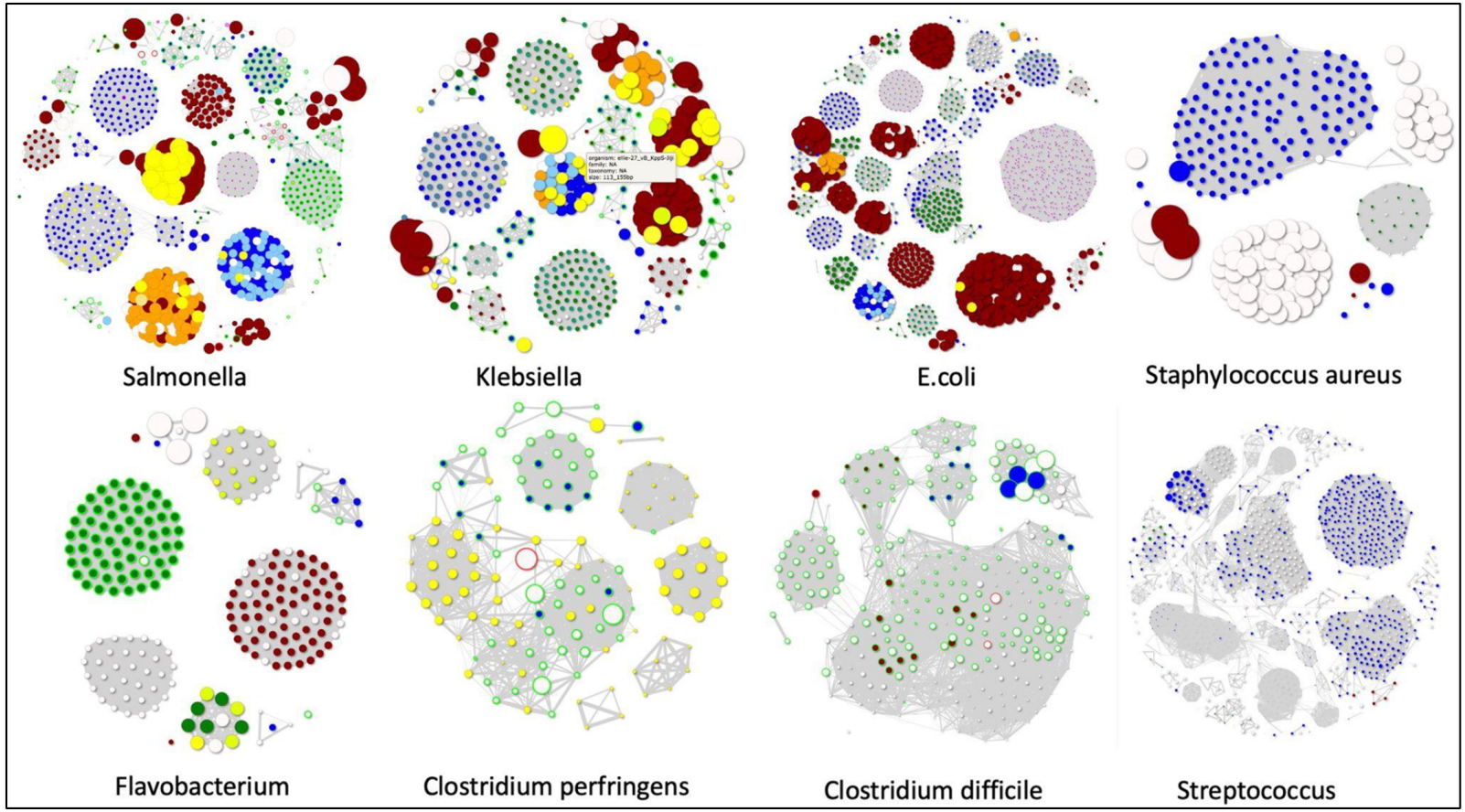


Figure 8: The *Klebsiella* and *E. coli* clouds alongside clouds for several other important pathogens. Ultimately, we hope to use this approach so see if the biology of the phages correlates with these groupings. Clearly the number of phages within each group dictates aspects of the cloud formation, but regardless of this we can see features such as connectivity, largest number of representations and number of discrete clouds all of which will form starting points to understanding dynamics specific phage groups.

Overall patterns within clouds

Schematic phage clouds for several common bacterial pathogens of humans and other animals are shown in Figure 8. Clearly phage clouds that contain a lot of species have a difference in density to those with relatively few representatives. We are currently investigating if we can identify patterns within host specific phage cloud space and apply that information to the phage cloud space from unrelated hosts.

LESSONS FROM ANIMAL STUDIES

Phages can amplify *in situ*

As stated in our Systematic Reviews of clinical phage use, there is little data available on the ability of phages to replicate *in vivo*, which impacts our ability to determine the appropriate dose to use within our human trials. Studies in animals can inform us of dose responses and show us how the dose changes the efficacy. Furthermore, as mentioned above, very little work has been carried out in large animals and thus pig trials can be particularly informative in terms of the pharmacodynamics and pharmacokinetics.

From the pig data (Figure 9A and B) it was clear that when phages were given to the animals who were then challenged with *Salmonella*, there was a reduction of bacterial numbers and an increase in phage amplification throughout their digestive tract. In contrast, when phages were given to the pigs in the absence of bacterial challenge the number of phages observed was consistently lower.

Our data show that ultimately it may be possible to add relatively small doses of the phages and demonstrate efficacy. During our study we added phages at 10^5 phage per gram within the pig feed. The selection of a low dose was dictated by the fact that we lost a significant amount of the phage titre following the industrial spray drying of the phages in order to be able to incorporate them into the animal feed. These low dosages but clear amplification are consistent with the way that phage preparations are given to patients in Georgia. In general a low titre, highly diverse phage mix is given to patients with the idea being that the correct phage within the mixture will amplify *in situ*.

Lower dosages are more effective than higher dosages

Although the pig work was a relatively small study, the data on *Salmonella* reduction and phage amplification provided us with the incentive to test the pages on a larger scale. We have subsequently carried out a large-scale chicken trial with 672 birds (Figure 9C). The main objective of this trial was to show safety and efficacy and determine the optimal phage dose. The most striking observation from our data was that the lowest phage dose was the most effective in terms of clearing *Salmonella* from the intestine of the birds. Here we have presented the data in terms of the amount of *Salmonella* bacteria that were shed from the chickens in terms of what could be recovered from sampling the pens (there were sixteen pens per treatment group). When data were analysed in terms of the number of colonies shed from individual birds, the same trend was apparent and the lowest dose (0.1x) was the most effective. The reason for this highly effective lower dose is unknown, but we hypothesise that it is related to the lower dose having a lower rate of bacterial killing which would allow higher levels of localised replication and infection prior to clearance by the host immune response.

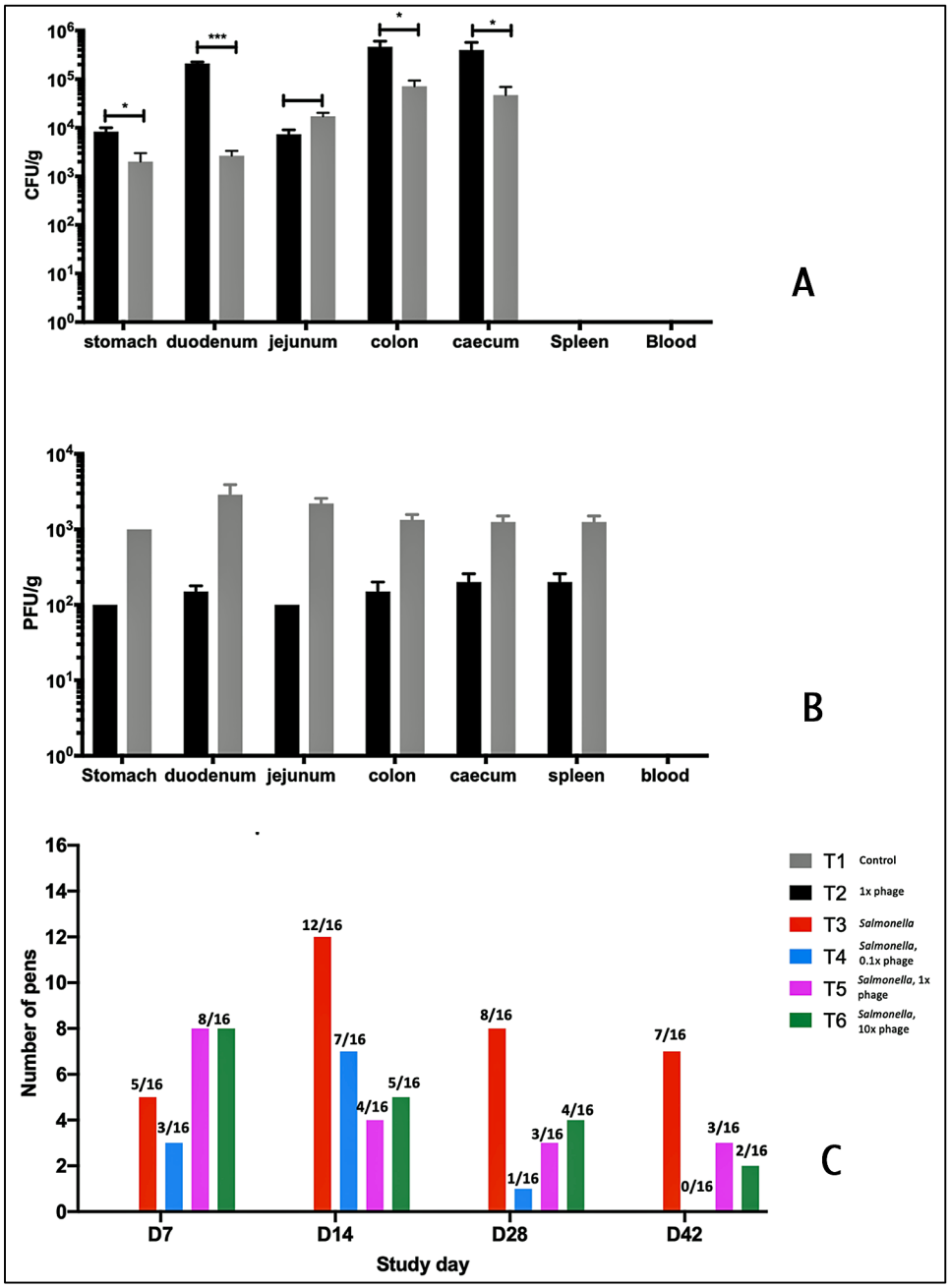


Figure 9: A) the number of bacteria isolated from the different regions of a pig digestive five days post challenge (*Thanki* et al., 2022). In the sample group that were given phages, significantly fewer bacteria were found in the stomach, duodenum, colon and caecum than were found in the control group. **B)** the number of phages in each section of the gut, with the animals given the bacterial challenge shown in grey. In all cases, the animals that received bacterial challenge had a greater number of phages in the sample. **C)** the data from a poultry study where phages were given in feed to poultry and the bacteria enumerated from the chicken pens. These data show that phages reduce the spread of infection by reducing the bacterial load shedding from the animals. At the lowest phage dose (0.1x), no bacteria were found in any pen. The other two doses (1x and 10x) also had a markedly good impact on the amount of *Salmonella* that can be recovered after 42 days.

Resistance

One of the knowledge gaps that we recognised from the systematic reviews was information on bacterial resistance. Clearly there is a lot of data on this within the literature, but most of it is from *in vitro* work. We isolated bacterial colonies from the multiple days up to 42 of the study and in no cases did we find examples of bacteria that were resistant to our phages. Forty-two days is not a particularly long time-frame but it is encouraging that we did not see resistance on this time scale. Future work will be needed in order to confirm that specific phages do not drive high resistance rates. Clearly, it is of paramount importance to ensure that wedo not repeat the mistakes of the past and breed phage resistance in these settings that would make human based treatments ineffective

CLINICAL TRIAL

The overall aim of the current pre-clinical UTI based studies within our group is to provide the necessary data that will allow us to conduct a clinical trial in humans. We plan to conduct a phase I/II multi-centre clinical trial with participants who have known recurrent *E.coli* and *Klebsiella* UTIs.

Our pre-clinical work is currently in progress and involves the use of a murine UTI model to provide preliminary efficacy and safety data of our defined phage cocktail in an *in vivo* model. The model will involve the direct intraurethral administration of phages to mimic how they would be used in humans where they would be catheterised directly into the bladder. We are using relevant clinical strains of *E. coli* and *Klebsiella* that have already been well characterised within murine models, and are using our optimised phage cocktails. In addition to the murine work, we will also assess human urinary epithelial cell toxicity and immune responses.

In the USA, there is an interesting clinical trial that is currently recruiting patients, being carried out by Adaptive Phage Therapeutics - APT (NCT04287478). There are similarities with our work, in that researchers involved with this trial will treat UTIs caused by *E.coli* and *Klebsiella*. The main difference is that they will provide 'personalised' phage therapy as opposed to the 'off-the shelf' product we will be using. To ensure our trial provides data on efficacy, our inclusion criteria will ensure only participants who have bacterial isolates that are sensitive to our defined phage cocktail. Another difference is that the APT trial will compare two routes of administration, intravesical and intravenous, while our clinical trial will assess the intravesical route only.

REGULATION

In a recent report published by the Antibacterial Resistance Leadership Group (ARLG) based in the USA, it was indicated that the compassionate use of phages is a viable regulatory pathway for the use of phages to treat patients who have few options left. The ARLG endorsed the collection of systematic data on patients who receive phage therapy through this access pathway until regulatory bodies approve a licensed phage therapy (*Suh* et al., 2022).

At present in the UK, there are no licensed phage products and no specific

regulatory frameworks for the use of phage-based products in humans, so compassionate use is the only way phage therapy can be administered to a patient. In the UK, phage therapy can be prescribed to a patient on a 'named patient basis'. This type of prescription is only carried in certain circumstances when particular criteria are met and it considers the special needs of an individual patient. Pathways established to drive innovation in medicine by the UK government and within the UK's National Health Service (NHS) such as the Promising Innovative Medicine Designation (PIM) and the Innovative Licensing and Access Pathway (ILAP) could potentially provide routes for licensing phage-based therapies for compassionate use on a wider scale, though it is not yet clear how this would work.

Within the UK, following clinical trial data to support safety and efficacy of phage products for human therapeutic use will be regulated through the Medicines and Healthcare products Regulatory Agency - MHRA. It is clear that phage products used on a large-scale, and for compassionate use will have to be produced under GMP (Good Manufacturing Practice) conditions. Although there are no facilities currently available for this to occur within the UK, there is significant demand for this resource and growing interest in setting up such a facility that UK academics, doctors and companies producing phages for clinical trials can access. There are several ongoing initiatives that are actively working towards making phage therapy more accessible for clinicians and patients in the UK as well as facilitating discussions with key stakeholders on the potential for establishing a phage production facility in the UK. Globally such facilities can be accessed although costs can be prohibitive with a significantly large proportion of the resource required for a clinical trial being directed into GMP phage production.

CONCLUSIONS AND FINAL THOUGHTS

In this paper we have outlined why UTIs are a major problem and why there is a desperate need to develop novel treatments. We also summarised the reviews on the extensive body of literature that strongly suggests that phages are inherently safe. There is a caveat to this of course, which is that many aspects of toxicity have not been rigorously assessed and such studies are needed.

From our work we show that the earlier key phage traits can be integrated into a phage selection programme, the better. Simply identifying phages on the basis of their host range and virulence is likely to result in omitting useful phages. Our current approach may lead to identifying phages/phage cocktails which are good all-rounders; on the other hand it could be that a phage needs to have the ability to cope with stress such as a low oxygen environment, which will result in higher virulence than as measured under optimal conditions.

Our work on larger animals and within large scale settings., and mouse work using a UTI model, provides a pre-clinical framework for a human clinical trial of phage therapy for UTI

Importance of the long view

It is important to remember that we are building on a large body of literature assembled by our Georgian, Russian and Polish colleagues. In addition, there is more clinical interest than ever before. It is possible to go straight from phage isolation to genome sequencing.

Bacteriophage bioinformatics

There are many bioinformatic challenges that bacteriophage genomes present us. Some of our authors have a strong background in using bioinformatics to extract information from ancient life, complex metagenomes and unique evolutionary trajectories – we have combined forces to apply this knowledge to identify phage diversity. The extreme diversity of phage genomes limits our ability to identify the function of the majority of their putative open reading frames. This means that even genes that we know must be present within the genome such as major structural genes are not always identifiable. This may even extend to whole bacteriophage genomes which may not be recognisable either from metagenomic data sets or from whole bacterial genomes. To address this, we have written Phageboost that looks to find novel phages from within bacterial genomes or metagenomes based on feature space rather than sequence similarity (*Sirén* et al., 2021).

Furthermore, the fact that they have no genes in common means that a comparative scheme such as that used to barcode or other forms of life based on shared ribosomal RNA is not available. To address this, we designed the graph-based phage cloud approach that we presented above to examine genome relationships. To identify phages suitable for therapy we exploited our knowledge of attributes that render phages unsuitable for therapy and developed Phageleads as screening tool (*Yukgehnaish* et al., 2022).

Molecular and structural biology

Another pertinent aspect of phage therapy is that unlike the case for previous generations of phage researchers, we can manipulate the genomes of an increasing number of phages. There are an increasing number of recombineering and selection approaches coming on-line, CRISPR based technologies for example are becoming more standard and synthetic biology and Gibson assemblies followed by approaches such as 'rebooting' in easier hosts means that phages can be manipulated to understand the relevance of key features and also potentially to expand and extend their properties. These approaches will be increasingly important to gain fundamental knowledge to expand phage properties.

Phage structural biology is likely to be of increasing importance to progress our understanding of therapeutically relevant phages. Again, new approaches such as Alphafold2 that predicts protein structures e.g. (*Dowah* et al., 2021; *Tunyasuvunakool* et al., 2021) will be of importance to predict key structures that can then be verified using standard approaches and direct biochemical and functional analysis. Furthermore, using cryo-electron microscopy and electron tomography to resolve phage protein and phage-host protein complexes are likely to be important in understanding how bacteriophages function.

Final thoughts inspired by patients

We have outlined the healthcare problem, the economic backdrop and the lack of availability of antimicrobials, particularly with respect to UTIs caused by *E. coli* and *Klebsiella*. We have also detailed our approaches to developing and testing phage sets. The length and multifaceted nature of this paper reflects the many aspects to phage therapy development. Whilst understanding the science and elucidating a mechanistic basis for why phages work is of paramount importance to developing phages in a sustainable and effective manner, it is important to remember that AMR infections in people is a real problem now and thus we need to expedite

phage development programmes to save lives and prevent misery.

LITERATURE

Abernethy, J., Gerver, S., Johnson, A., and Hope, R.: Thirty-day all-cause fatality subsequent to MRSA, MSSA and E. coli bacteraemia and C. difficile infection. (2016). DOI: 10.13140/RG.2.1.2804.1840

Antimicrobial Resistance Collaborators. (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet 399, 629-655 (2022).

Aranaga, C., Pantoja, L.D., Martínez, E.A., and Falco, A.: Phage Therapy in the Era of Multidrug Resistance in Bacteria: A Systematic Review. Int. J. Mol. Sci. 23, 4577 (2022).

Azevedo, M.M., Pina-Vaz, C., and Rodrigues, A.G.: The Role of Phage Therapy in Burn Wound Infections Management: Advantages and Pitfalls. J. Burn Care Res. 43, 336-342 (2022).

Budd, E., Cramp, E., Sharland, M., Hand, K., Howard, P., Wilson, P., Wilcox, M., Muller-Pebody, B., and Hopkins, S.: Adaptation of the WHO Essential Medicines List for national antibiotic stewardship policy in England: being AWaRe. J. Antimicrob. Chemother. 74, 3384-3389 (2019).

Caneiras, C., Lito, L., Melo-Cristino, J., and Duarte, A.: Community- and Hospital-Acquired Klebsiella pneumoniae Urinary Tract Infections in Portugal: Virulence and Antibiotic Resistance. Microorganisms 7, 138 (2019).

Cassini, A., Högberg, L.D., Plachouras, D., Quattrocchi, A., Hoxha, A., Simonsen, G.S., Colomb-Cotinat, M., Kretzschmar, M.E., Devleesschauwer, B., Cecchini, M., Ouakrim, D. A., Oliveira, T.C., Struelens, M.J., Suetens, C., Monnet, D.L., Burden of AMR Collaborative Group: Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. Lancet Infect. Dis. 19, 56-66 (2019).

Clarke, A.L., De Soir, S., and Jones, J.D.: The Safety and Efficacy of Phage Therapy for Bone and Joint Infections: A Systematic Review. Antibiotics (Basel) 9: 795 (2020).

Clokie, M.R.J., Blasdel, B.G., Demars, B.O.L., and Thomas Sicheritz-Pontén, T.: Rethinking phage ecology by rooting it within an established plant framework. PHAGE 1, 121-136 (2020).

Dąbrowska, K.: Phage therapy: What factors shape phage pharmacokinetics and bioavailability? Systematic and critical review. Med. Res. Rev. 39, 2000-2025 (2019).

Dąbrowska, K. and Abedon, S.T.: Pharmacologically Aware Phage Therapy: Pharmacodynamic and Pharmacokinetic Obstacles to Phage Antibacterial Action in Animal and Human Bodies. Microbiol. Mol. Biol. Rev. 83, e00012-19 (2019).

Day, M.J., Hopkins, K.L., Wareham, D.W., Toleman, M.A., Elviss, N., Randall, L., Teale, C., Cleary, P., Wiuff, C., Doumith, M., Ellington, M.J., Woodford, N., and Livermore, D.M.: Extended-spectrum β-lactamase-producing Escherichia coli in human-derived and foodchain-derived samples from England, Wales, and Scotland: an epidemiological surveillance and typing study. Lancet Infect. Dis. 19, 1325-1335 (2019).

Dedrick, R.M., Guerrero-Bustamante, C.A., Garlena, R.A., Russell, D.A., Ford, K., Harris, K., Gilmour, K.C., Soothill, J., Jacobs-Sera, D., Schooley, R.T., Hatfull, G.F., and Spencer, H.: Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant Mycobacterium abscessus. Nat. Med. *25*, 730-733 (2019).

Djebara, S., Maussen, C., De Vos, D., Merabishvili, M., Damanet, B., Pang, K., De Leenheer, P., Strachinaru, I., Soentjens, P., and Pirnay, J.-P.: Processing Phage Therapy Requests in a Brussels Military Hospital: Lessons Identified. Viruses 11, 265 (2019).

Doumith, M., Day, M., Ciesielczuk, H., Hope, R., Underwood, A., Reynolds, R., Wain, J., Livermore, D.M., and Woodford, N.: Rapid identification of major Escherichia coli sequence types causing urinary tract and

bloodstream infections. J. Clin. Microbiol. 53, 160-166 (2015).
Dowah, A.S.A., Xia, G., Ali, A.A.K., Thanki, A.M., Shan, J., Millard, A., Petersen, B., Sicheritz-Pontén, T., Wallis, R., and Clokie, M.R.J.: The structurome of a Clostridium difficile phage and the remarkable accurate prediction of its novel phage receptor-binding protein. bioRxiv 2021.07.05.451159 (2021).
El Haddad, L., Harb, C.P., Gebara, M.A., Stibich, M.A., and Chemaly, R.F.: A Systematic and Critical Review of Bacteriophage Therapy Against Multidrug-resistant ESKAPE Organisms in Humans. Clin. Infect. Dis. Jun 18, 167-178 (2019).
Genevière, J., McCallin, S., Huttner, A., Pham, T.T., and Suva, D.: A systematic review of phage therapy applied to bone and joint infections: an analysis of success rates, treatment modalities and safety. EFORT Open Rev. 6, 1148-1156 (2021).
Gordillo Altamirano, F.L. and Barr, J.J.: Phage Therapy in the Postantibiotic Era. Clin. Microbiol. Rev. 32, e00066-18 (2019).
Gorrie, C.L., Mirceta, M., Wick, R.R., Judd, L.M., Wyres, K.L., Thomson, N.R., Strugnell, R.A., Pratt, N.F., Garlick, J.S., Watson, K.M., Hunter, P.C., McGloughlin, S.A., Spelman, D.W., Jenney, A.W.J., and Holt, K.E.: Antimicrobial-Resistant Klebsiella pneumoniae Carriage and Infection in Specialized Geriatric Care Wards Linked to Acquisition in the Referring Hospital. Clin. Infect. Dis. 67, 161-170 (2018).
Grime, J.P.: Plant Strategies and Vegetation Processes. John Wiley & Sons Inc., Hoboken, New Jersey, USA (1979).
Guengerich, F.P.: Mechanisms of drug toxicity and relevance to pharmaceutical development. Drug Metab. Pharmacokinet. 26, 3-14 (2011).
Haines, M.E.K., Hodges, F.E., Nale, J.Y., Mahony, J., van Sinderen, D., Kaczorowska, J., Alrashid, B., Akter, M., Brown, N., Sauvageau, D., Sicheritz-Ponten, T., Thanki, A.M., Millard, A.D., Galyov, E.E., and Clokie, M.R.J.: Analysis of selection methods to develop novel phage therapy cocktails against antimicrobial resistant clinical isolates of bacteria. Front. Microbiol. 12, 613529 (2021).
Hancock, V., Dahl, M., and Klemm, P.: Abolition of biofilm formation in urinary tract Escherichia coli and Klebsiella isolates by metal interference through competition for fur. Appl. Environ. Microbiol. 76, 3836-3841 (2010).
Hodges, F.E., Sicheritz-Pontén, T., and Clokie, M.R.J.: The effect of oxygen availability on bacteriophage infection: A review. PHAGE 2, 16-25 (2021).
Jończyk, E., Kłak, M., Międzybrodzki, R., and Górski, A.: The influence of external factors on bacteriophages--review. Folia Microbiol. (Praha) 56, 191-200) (2011).
Ssekatawa, K., Byarugaba, D.K., Kato, C.D., Wampande, E.M., Ejobi, F., Tweyongyere, R., and Nakavuma, J.L: A review of phage mediated antibacterial applications, Alexandria J. Med. 57, 1-20 (2021).
Kwiatek, M., Parasion, S., and Nakonieczna, A.: Therapeutic bacteriophages as a rescue treatment for drug-resistant infections - an in vivo studies overview. J. Appl. Microbiol. 128, 985-1002 (2020).
Liu, D., Van Belleghem, J.D., de Vries, C.R., Burgener, E., Chen, Q., Manasherob, R., Aronson, J.R., Amanatullah, D.F., Tamma, P.D., and Suh, G.A.: The Safety and Toxicity of Phage Therapy: A Review of Animal and Clinical Studies. Viruses 13, 1268 (2021).
Łusiak-Szelachowska, M., Międzybrodzki, R., Drulis-Kawa, Z., Cater, K., Knežević, P., Winogradow, C., Amaro, K., Jończyk-Matysiak, E., Weber-Dąbrowska, B., Rękas, J., and Górski, A.: Bacteriophages and antibiotic interactions in clinical practice: what we have learned so far. J. Biom. Sci. 29, 23 (2022).
Mantle, S: Reducing HCAI-What the Commissioner needs to know. NHS England, UK, 2015: https://www.england.nhs.uk/wp-content/uploads/2015/04/10-amr-lon-reducing-hcai.pdf
Medina, M. and Castillo-Pino, E.: An introduction to the epidemiology and burden of urinary tract infections. Ther. Adv. Urol. 11, 1756287219832172 (2019).
Nale, J.Y. and Clokie, M.R.J.: Preclinical data and safety assessment of phage therapy in humans. Curr. Opin. Biotechnol. 68, 310-317 (2021).

Naylor, N.R., Pouwels, K.B., Hope, R., Green, N., Henderson, K.L., Knight, G.M., Atun, R., Robotham, J.V., and Deeny, S.R.: The health and cost burden of antibiotic resistant and susceptible Escherichia coli bacteraemia in the English hospital setting: A national retrospective cohort study. PLoS One 14: e0221944 (2019).

NHS England, UK: Commissioning for Quality and Innovation (CQUIN), 2022/23: https://www.england.nhs.uk/publication/combined-ccg-icb-and-pss-commissioning-for-quality-and-innovation-cquin-guidance/

O'Neill, J.: Tackling drug-resistant infections globally: final report and recommendations (2016). Government of the United Kingdom: https://apo.org.au/node/63983

Özal, D., Arndt, A., and Thomé, M.: Bacteriophages and related endolysins for reduction of microorganisms in the human body - a systematic review. GMS Hyg. Infect. Control 17, Doc01 (2022).

Pires, D.P., Costa, A.R., Pinto, G., Meneses, L., and Azeredo, J.: Current challenges and future opportunities of phage therapy. FEMS Microbiol. Rev. 44, 684-700 (2020).

Pirnay, J.-P., De Vos, D., Verbeken, G., Merabishvili, M., Chanishvili, N., Vaneechoutte, M., Zizi, M., Laire, G., Lavigne, R., Huys, I., Van den Mooter, G., Buckling, A., Debarbieux, L., Pouillot, F., Azeredo, J., Kutter, E., Dublanchet, A., Górski, A., and Adamia, R.: The phage therapy paradigm: prêt-à-porter or sur-mesure? Pharm. Res. 28, 934-937 (2011).

Podlacha, M., Grabowski, Ł., Kosznik-Kawśnicka, K., Zdrojewska, K., Stasiłojć, M., Węgrzyn, G., and Węgrzyn, A.: Interactions of Bacteriophages with Animal and Human Organisms-Safety Issues in the Light of Phage Therapy. Int. J. Mol. Sci. 22, 8937 (2021).

Principi, N., Silvestri, E., and Esposito, S.: Advantages and Limitations of Bacteriophages for the Treatment of Bacterial Infections. Front. Pharmacol. 10, 513 (2019).

Pujades-Rodriguez, M., West, R.M., Wilcox, M.H., and Sandoe, J.: Lower Urinary Tract Infections: Management, Outcomes and Risk Factors for Antibiotic Re-prescription in Primary Care. EClinicalMedicine 14, 23-31 (2019).

Rahimzadeh, G., Saeedi, M.,Farshidi, F., and Reza, M.S. Phage Therapy in Treatment of Gram-negative Bacterial Infections: A Systematic Review. (Persian). J. Mazandaran Univ. Med. Sci. 28, 203-212 (2018).

Rangel-Pineros, G., Millard, A., Michniewski, S., Scanlan, D., Sirén, K., Reyes, A., Petersen, B., Clokie, M.R.J., and Sicheritz-Pontén, T.: From Trees to Clouds: PhageClouds for Fast Comparison of ~640,000 Phage Genomic Sequences and Host-Centric Visualization Using Genomic Network Graphs. PHAGE 2, 194-203 (2021).

Saperkin, N.V., Kovalishena, O.V., Kvashnina, D.V., Ruizendaal, E., and Scholten, R.: Efficiency of phage therapy in humans: systematic review. J. Infectol. 11, 19-30 (2019). (In Russ.)

Shrivastava, S., Shrivastava, P., and Ramasamy, J.: World health organization releases global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. J. Med. Soc. 32, 76-77 (2018).

Sirén, K., Millard, A., Petersen, B., Gilbert, M.T.P., Clokie, M.R.J., and Sicheritz-Pontén, T.: Rapid discovery of novel prophages using biological feature engineering and machine learning. NAR Genom. Bioinform. 3, Iqaa109 (2021).

Steele, A., Stacey, H.J., de Soir, S., and Jones, J.D.: The Safety and Efficacy of Phage Therapy for Superficial Bacterial Infections: A Systematic Review. Antibiotics (Basel) 9, 754 (2020).

Storms, Z.J., Teel, M.R., Mercurio, K., and Sauvageau, D.: The Virulence Index: A Metric for Quantitative Analysis of Phage Virulence. PHAGE 1, 27-36 (2020).

Suh, G.A., Lodise, T.P., Tamma, P.D., Knisely, J.M., Alexander, J., Aslam, S., Barton, K.D., Bizzell, E., Totten, K.M.C., Campbell, J.L., Chan, B.K., Cunningham, S.A., Goodman, K.E., Greenwood-Quaintance, K.E., Harris, A.D., Hesse, S., Maresso, A., Nussenblatt, V., Pride, D., Rybak, M.J., SDund, Z., van Duin, D., Van Tyne, D., Patel, R., Antibacterial Resistance Leadership Group: Considerations for the Use of Phage Therapy in Clinical Practice. Antimicrob. Agents

Chemother. 66, e0207121(2022).
Thanki, A.M., Mignard, G., Atterbury, R.J., Barrow, P., Millard, A.D., and Clokie, M.R.J.: Prophylactic Delivery of a Bacteriophage Cocktail in Feed Significantly Reduces Salmonella Colonization in Pigs. Microbiol. Spectr. 10, e0042222 (2022).
Thomas, R.E. and Thomas, B.C.: Reducing Biofilm Infections in Burn Patients' Wounds and Biofilms on Surfaces in Hospitals, Medical Facilities and Medical Equipment to Improve Burn Care: A Systematic Review. Int. J. Environ. Res. Public Health 18, 13195 (2021).
Tiwari, R., Dhama, K., Kumar, A., Rahal, A., and Kapoor, S.: Bacteriophage therapy for safeguarding animal and human health: a review. Pak. J. Biol. Sci. 17, 301-315 (2014).
Townsend, E.M., Kelly, L., Gannon, L., Muscatt, G., Dunstan, R., Michniewski, S., Sapkota, H., Kiljunen, S.J., Kolsi, A., Skurnik, M., Lithgow, T., Millard, A.D., and Jameson, E. Isolation and Characterization of Klebsiella Phages for Phage Therapy. PHAGE 2, 26-42 (2021).
Tsonos, J., Vandenheuvel, D., Briers, Y., De Greve, H., Hernalsteens, J.-P., and Lavigne, R:. Hurdles in bacteriophage therapy: deconstructing the parameters. Vet. Microbiol. 171, 460-469 (2014).
Tunyasuvunakool, K., Adler, J., Wu, Z., Green, T., Zielinski, M., Žídek, A., Bridgland, A., Cowie, A., Meyer, C., Laydon, A., Velankar, S., Kleywegt, G.J., Bateman, A., Evans, R., Pritzel, A., Figurnov, M., Ronneberger, O., Bates, R., Kohl, S.A.A., Potapenko, A., Ballard, A.J., Romera-Paredes, B., Nikolov, S., Jain, R., Clancy, E., Reiman, D., Petersen, S., Senior, A.W., Kavukcuoglu, K., Birney, E., Kohli, P., Jumper, J., and Hassabis, D.: Highly accurate protein structure prediction for the human proteome. Nature 596, 590-596 (2021).
UK Health Security Agency: English surveillance programme for antimicrobial utilisation and resistance (ESPAUR) Report 2020 to 2021 (2021). https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/1069632/espaur-report-2020-to-2021-16-Nov-FINAL-v2.pdf
Uyttebroek, S., Chen, B., Onsea, J., Ruythooren, F., Debaveye, Y., Devolder, D., Spriet, I., Depypere, M., Wagemans, J., Lavigne, R., Pirnay, J.-P., Merabishvili, M., De Munter, P., Peetermans, W. E., Dupont, L., Van Gerven, L., and Metsemakers, W.-J.: Safety and efficacy of phage therapy in difficult-to-treat infections: a systematic review. Lancet Infect. Dis. 22, e208-e220 (2022).
Van Belleghem, J.D., Clement, F., Merabishvili, M., Lavigne, R., and Vaneechoutte, M.: Pro- and anti-inflammatory responses of peripheral blood mononuclear cells induced by Staphylococcus aureus and Pseudomonas aeruginosa phages. Sci. Rep. 7, 8004 (2017).
Walter, N., Deng, L., Brochhausen, C., Alt, V., and Rupp, M.: Treatment of bone and periprosthetic infections with bacteriophages : A systematic review. Orthopade 51, 138-145 (2022).
World Health Organization (WHO): Prioritization of pathogens to guide discovery, research and development of new antibiotics for drug-resistant bacterial infections, including tuberculosis (2017). https://www.who.int/publications/i/item/WHO-EMP-IAU-2017.12
Yukgehnaish, K., Rajandas, H., Parimannan, S., Manickam, R., Marimuthu, K., Petersen, B., Clokie, M.R.J., Millard, A., and Sicheritz-Pontén, T.: PhageLeads: Rapid Assessment of Phage Therapeutic Suitability Using an Ensemble Machine Learning Approach. Viruses 14, 342 (2022).
Zeng, Z., Zhan, J., Zhang, K., Chen, H., and Cheng, S.: Global, regional, and national burden of urinary tract infections from 1990 to 2019: an analysis of the global burden of disease study 2019. World J. Urol. 4), 755-763 (2022).

MYCOBACTERIOPHAGES: DIVERSITY, DYNAMICS, AND THERAPEUTIC POTENTIAL

GRAHAM F. HATFULL

Department of Biological Sciences, University of Pittsburgh,
Pittsburgh PA 15260, USA

SUMMARY

Mycobacteriophages are viruses that infect *Mycobacterium* hosts, including the pathogens *Mycobacterium tuberculosis* and *Mycobacterium abscessus*, as well as environmental strains such as *Mycobacterium smegmatis*. A large collection of phages isolated on *M. smegmatis* – of which more than 2,000 have been completely sequenced and annotated – provides a high-resolution view of viral diversity, origins, and the mechanisms of viral evolution. This diversity underlies extensive variations in phage life cycles, gene expression and its regulation, and is driven by the highly dynamic relationship between bacteria and their viral invaders. Many *Mycobacterium* infections in humans – especially those caused by multidrug-resistant tuberculosis or non-tuberculous mycobacteria (NTM) strains such as *M. abscessus* – are very challenging to treat with currently available antibiotic regimens, and mycobacteriophages present a plausible option for patients with no further traditional treatment options. A series of case studies using phages to treat a variety of NTM infections provides insights into the considerable opportunities as well as the challenging limitations of this therapeutic strategy.

INTRODUCTION

The bacteriophage population is vast, highly dynamic, and old (*Hendrix*, 2002). It is perhaps not surprising then that it is highly diverse genetically, shaped by billions of years of variation, selection, and pervasive genetic exchange (*Hatfull* and *Hendrix*, 2011). Bacteria are under constant attack by lytically growing viruses, and must evolve resistance to survive, while the phages must co-evolve to infect sensitive bacterial hosts (*Hampton* et al., 2020). These bacterial-phage dynamics have dominated microbial evolution for perhaps three billion years and have strongly influenced nearly all aspects of bacterial and bacteriophage genomes (*Bernheim* and *Sorek*, 2020).

To characterize bacteriophages, it is not uncommon to focus on one or a relatively small number of bacterial hosts, which simplifies the technical approaches, and it is expected that general principles learned this way will be applicable to phages of other bacterial hosts (*Hatfull*, 2015a). Different bacterial systems offer various attributes, and several years ago we chose to isolate and study phages of *Mycobacterium smegmatis*, a non-pathogenic relative of the human pathogens *Mycobacterium tuberculosis* and *Mycobacterium abscessus* (among others) (*Hatfull*, 2006). There are three key attributes favouring the development of this system. First, mycobacteriophages

(viruses of *Mycobacterium*) can offer insights into viral diversity, evolution, and origins, and represents a notable departure from well-studied model organisms such as *Escherichia coli*. Secondly, mycobacteriophages are a source of broadly applicable genetic tools, and can thus advance the genetic analysis of important pathogens such as *M. tuberculosis*, which grows very slowly and for many years was genetically intractable (*Jacobs*, 2000). Third, mycobacteriophages have potential utilities clinically for both diagnostic and therapeutic applications. Bacteriophage discovery and genomics have also proven to constitute a terrific platform for advancing science education, and course-based implementations of mycobacteriophage isolation and characterization have strongly impacted the phage science while inspiring many fledgling student researchers (*Hatfull*, 2015b). Here, I will discuss some of the key advances in our understanding of the diversity, dynamics, and therapeutic potential of mycobacteriophages.

MYCOBACTERIOPHAGE DIVERSITY

Mycobacteriophage isolation and genomics

Phages of *Mycobacterium* hosts were first isolated over 60 years ago, motivated in part by finding potential tools for typing of clinical isolates of *Mycobacterium*, particularly *M. tuberculosis* (*Snider* et al., 1984; *Good* and *Mastro*, 1989; *Cater* and *Redmond*, 1961; *Murohashi* et al., 1963). However, the faster growing and non-pathogenic *M. smegmatis* was shown to be a useful host for phage isolation, and several phages were characterized by host range and electron microscopy (*Takeya* et al., 1959a, 1959b, 1961). These early studies in the pre-genomic era showed that there was considerable variation in host range and morphotypes, and while a multitude of other features were described, their evolutionary relationships remained elusive (*Mizuguchi*, 1984).

The first mycobacteriophage with a completely sequenced genome was that of phage L5 (*Hatfull* and *Sarkis*, 1993), a temperate double-stranded DNA tailed phage of *M. smegmatis* isolated in Japan (*Doke*, 1960). This fuelled a series of further studies to characterize the phage, but also prompted a deeper dive into comparative mycobacteriophage genomics. Over the next decade a dozen more mycobacteriophages were sequenced (*Pedulla* et al., 2003), revealing that the overall diversity was indeed substantial and that many more phages would need to be characterized to provide a higher resolution genomic view. This was a major impetus for the development of integrated research-education programs that served the dual purposes of expanding the repertoire of sequenced phage genomes, while also providing authentic discovery-rich research experiences for novice scientists, including high school and undergraduate students (*Hanauer* et al., 2006). Initially, the Phage Hunters Integrating Research and Education (PHIRE) program was established to run locally at the University of Pittsburgh (*Hatfull* et al., 2006) but was subsequently expanded nationally in the US (but with some international participants) as the Science Education Alliance Phage Hunters Advancing Genomics and Evolutionary Science (SEA-PHAGES) (*Hanauer* et al., 2017, *Jordan* et al., 2014); both programs are funded by the Howard Hughes Medical Institution. The SEA-PHAGES

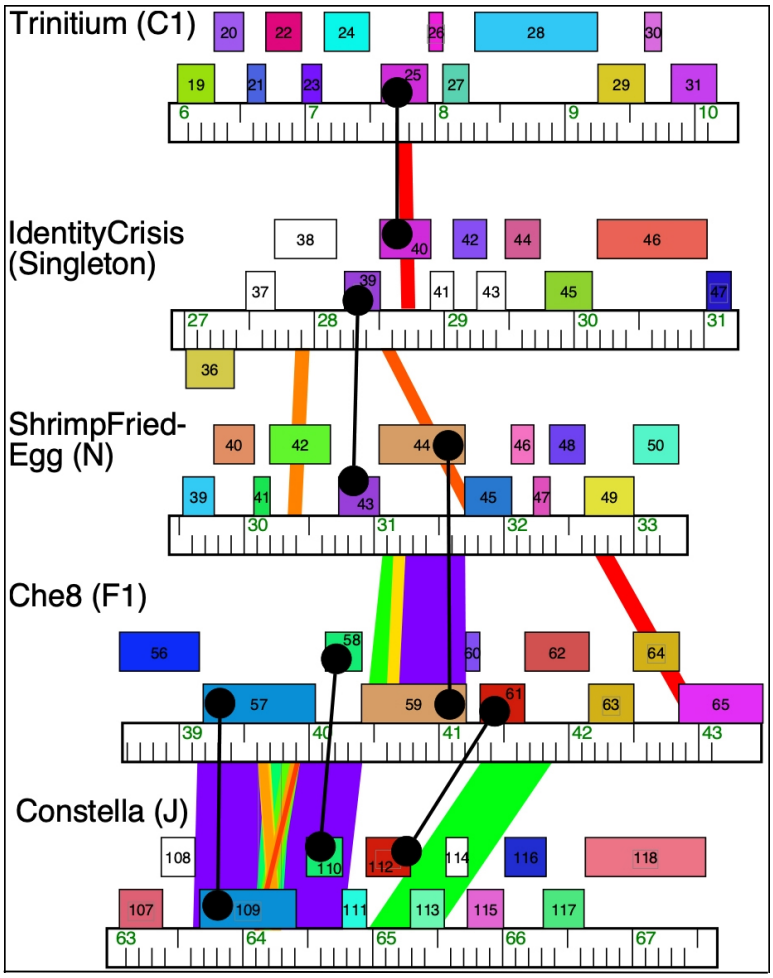


Figure 1: Genetic mosaicism in mycobacteriophage genomes.
Short segments of five phage genomes are shown, with their phage name and cluster/subcluster/singleton designations in parentheses. Genes are shown as coloured boxes either above or below the genome rulers, indicated rightwards and leftwards transcription, respectively, with gene numbers within the boxes. Genes in the same family are coloured similarly, and dumbbell lines are shown linking homologues in different genomes that share amino acid sequence similarity. Nucleotide sequence similarity is shown as colour-spectrum shading between the genomes, with violet being the most similar, and red being the least similar above a threshold BlastN value of 10^{-4}. Note that Trinitium *25* and IdentityCrisis *40* are related (50% amino acid identity) but are situated in different genomic contexts with different genes to their left and to their right. Che8 (F1) and Constella (J) have several genes in common in this region and note the segment of internal repeats reflected in the nucleotide similarity between Che8 *57* and Constella *109*.

program is large with over 170 participating institutions, and over 5,500 students each year. As a consequence, a very large collection of over 20,000 individual phages isolated from environmental samples has developed, of which over 4,000 are completely sequenced and annotated (*Hatfull*, 2020). Over 10,000 of these phages were isolated using *M. smegmatis*, of which over 2,000 are completely sequenced; the other phages were isolated using other bacteria within the phylum Actinobacteria and will not be

discussed in detail here. The complete collection is described at https://phagesdb.org.

Mycobacteriophage comparative genomics

How best to present the mycobacteriophage diversity? Early on in the genomic era it became apparent that groups of evidently related phages emerged, and that they shared little or no sequence similarity to phages outside their group (*Pedulla* et al., 2003, *Hatfull* et al., 2006, 2010). A system was therefore introduced in which sequence-related phages are grouped into 'clusters' (designated Cluster A, B, C etc.). Phages with no close relatives are designated as 'singletons'. When the collection was small, this classification was simple, because related phages were usually very similar at the nucleotide sequence level over large spans of their genomes, whereas phages in different clusters were completely different at the sequence level, often sharing few if any genes with shared amino acid sequences (*Hatfull* et al., 2010).

As the phage collection grew, a key architectural feature emerged, in that phage genomes are pervasively mosaic; that is, they are composed of genome segments – often single genes – that are exchanged among the phages, presumably by illegitimate or non-sequence-directed recombination (*Hendrix* et al., 1999; *Pedulla* et al., 2003). Thus, individual genes – or variants of genes – may be found in different genomes but in different contexts, flanked by different and unrelated genes (Figure 1). As a consequence, the expectation is that with deeper sampling of the phage population, the boundaries between clusters would become less clear, and that a continuum of diversity would emerge, albeit with unequal representation caused by isolation biases and differences in abundance of different phage types (*Pope* et al., 2015). This was indeed observed and warranted revision of the parameters used for cluster assignations. Currently, a value of 35% pairwise shared gene context is used for cluster inclusion, although this is essentially arbitrary, and does not reflect any underlying biological process (*Pope* et al., 2017). In fact, it is not hard to find pairwise shared gene content values straddling the 35% threshold value (*Mavrich* and *Hatfull*, 2017). A further finding is that there is often considerable intra-cluster diversity, and sometimes this appears sufficiently structured as to warrant division into 'subclusters' (i.e. Subcluster A1, A2, A3, within the broader Cluster A phages) (*Hatfull* et al., 2010).

Currently, the >2000 sequenced mycobacteriophages are grouped into 31 clusters and seven singletons, and a network phylogeny is shown in Figure 2. The largest by far is Cluster A with 722 members grouped into 20 subclusters, but several (Clusters U, V, X, Y, Z, AA, AB, AC, AD, AE) have five or fewer members (https://phagesdb.org/), together with the seven singletons. The sampling would thus seem incomplete, and there are likely more and different phages to be recovered from environmental samples, but the overall heterogeneity means that these are isolated with decreasing frequency. Clusters/subclusters often have common features and behaviours, including lifestyles and host ranges (*Mavrich* and *Hatfull*, 2017). For example, over 50% of the cluster/singleton set are predominantly temperate, and have genomic components such as repressor genes, integrases, or partitioning cassettes. However, it is not uncommon for lytic phages to be isolated that group within a temperate cluster but have lost their repressor gene and can no longer form lysogens (*Ford* et al., 1998). A simple explanation is that obligatorily lytic

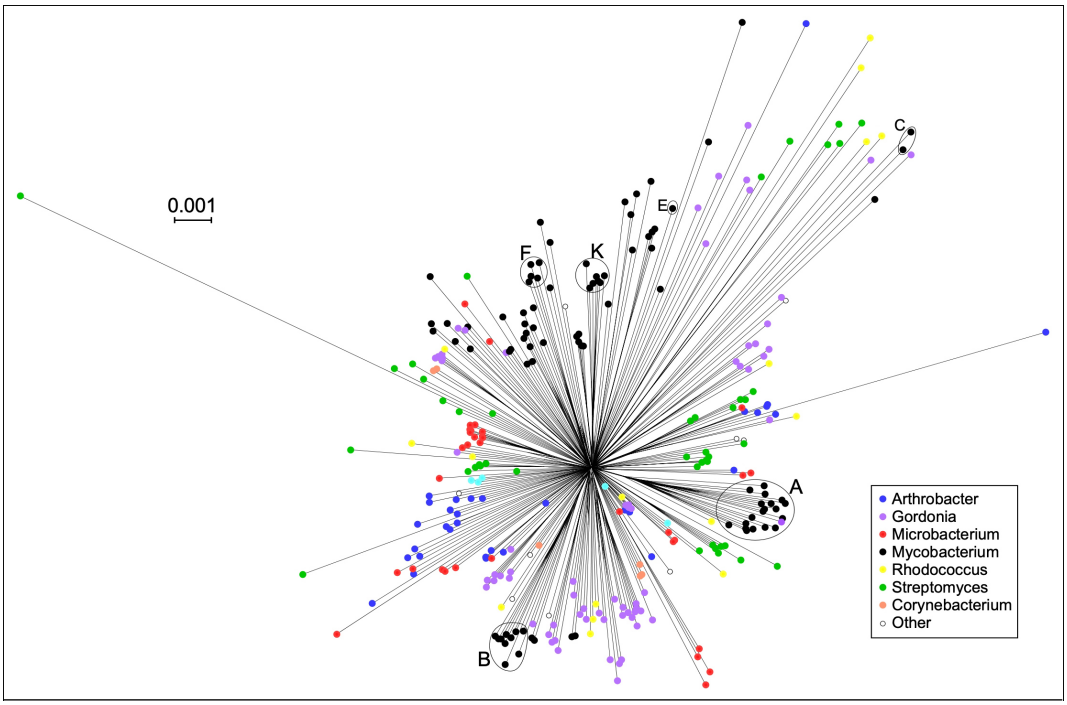


Figure 2: A network phylogeny of actinobacteriophages.
A randomly chosen phage from each subcluster and non-subclustered cluster together with the singletons were compared by their gene contents and the relationships displayed using SplitsTree (*Huson*, 1998). Each phage node is indicated by a coloured circle indicating the genus of the bacterial host used for isolation. Clusters containing 100 or more individual phages are circled and the cluster indicated. (Reproduced with permission from *Hatfull*, 2020).

phages form larger and clearer plaques and thus are easier to identify and to choose for purification during the isolation process. Cluster/subcluster designation can also correlate with host range, as demonstrated by the abilities of these phages to infect either *M. tuberculosis*, or other strains of *M. smegmatis* (*Jacobs-Sera* et al., 2012; *Guerrero-Bustamante* et al., 2021).

MYCOBACTERIOPHAGE LIFE CYCLES

As noted above, temperate phages are common among mycobacteriophages. These temperate phages are capable of cycles of lytic growth, similar to obligatorily lytic phages, but differ in that they can also enter the state of lysogeny upon infection. Transcriptomic analyses of lytic growth show that there are predominantly two patterns of transcription (*Gentile* et al., 2019, *Dedrick* et al., 2013, 2017b, 2019b). Soon after infection, early lytic gene expression is observed, which continues for about 20-30 minutes, then late lytic expression begins and continues until lysis about three hours after infection. The early lytic genes typically include those that are not directly involved in virion structure and assembly, functionally defined genes for DNA metabolism and regulation, but also a multitude of genes of unknown function. These

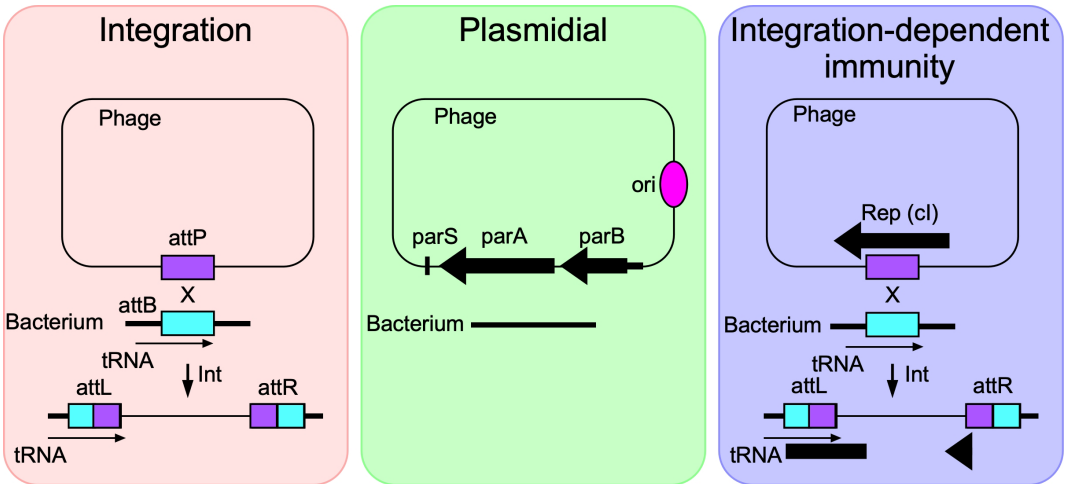


Figure 3: Systems for establishment of lysogeny and prophage maintenance.
When the linear viral DNA of a temperate phage is injected into the cell, the genome circularizes and either integrates ('Integration') or replicates as an extrachromosomal circle ('Plasmidial'). In the integration process, the phage-encoded integrase (Int) catalysed site-specific recombination between phage and bacterial attachment sites (*attP* and *attB* respectively) to form an integrated prophage flanked by attachment junctions *attL* and *attR*. The *attB* sites used by tyrosine-family integrases often overlap a host tRNA gene. Plasmidial phages do not integrate but replicate extrachromosomally from a phage origin of replication (ori) and encode partitioning systems (*parABS*) to prevent loss at cell division. Some phages use an unusual integration system ('Integration-dependent immunity') in which the *attP* site is located within the repressor gene (Rep, cI) of the temperate phage. Integration 'breaks' the repressor gene, separating its 5' and 3' parts in the prophage. The viral form of the repressor is inactive due to a C-terminal tag for proteolytic degradation, which is absent from the prophage-encoded repressor and can actively confer superinfection immunity.

genes are typically under the control of a single early lytic promoter, which is recognized by the host RNA polymerase, and in temperate phages is under control of the phage repressor (*Nesbit* et al., 1995, *Brown* et al., 1997). The late lytic genes are comprised of the virion structure and assembly genes, including those for DNA packaging and capsid and tail assembly. During late lytic growth, the lysis genes are also expressed, leading to cell rupture and the release of progeny particles (*Payne* et al., 2009; *Payne* and *Hatfull*, 2012).

For temperate phages, infection can result in either entry into lytic growth or the establishment of lysogeny. The frequencies with which these pathways are followed varies enormously for different phages and conditions, and lysogeny can result from as few as 5% of infections or as high as over 90% of infections. The molecular determinants of the decision outcomes are unclear for most mycobacteriophages, although they are well established for model systems such as phage lambda (*Ptashne*, 1987). Lysogeny itself, however, results from expression of the repressor protein and its down-regulation of the early lytic genes. Lysogens are characteristically immune to superinfection by the same phage, a phenomenon in which the prophage-expressed repressor down-regulates any newly-introduced genomes of the same phage (*Donnelly-Wu* et al., 1993). For most temperate mycobacteriophages, a prophage is established by site-specific

integration of the phage genome into the host chromosome (Figure 3), mediated by the phage-encoded integrase (*Lee* et al., 1991). However, some mycobacteriophages (all within Cluster A) do not integrate, and instead establish extrachromosomal 'plasmidial' prophages, which replicate at low copy number and encode partitioning systems to negate prophage loss at cell division (*Wetzel* et al., 2020) (Figure 3).

Mycobacteriophages use a variety of mechanisms for prophage integration and the establishment of lysogeny. Two main types of integration systems are prevalent, represented by the tyrosine-integrases (Int-Y) and serine-integrases (Int-S). Both catalyse site-specific recombination between a phage attachment site (*attP*) and a bacterial attachment site (*attB*) which share a short region of sequence identity (3-45 bp; the 'common core') within which strand exchange occurs. Recombination results in formation of an integrated prophage flanked by *attL* and *attR* sites (Figure 3). The integrase can also mediate excisive recombination by *attL* x *attR* exchange although this typically requires a phage-encoded Recombination Directionality Factor (RDF) (*Lewis* and *Hatfull*, 2001). Most Int-Y systems have common cores 25-45 bp long, and thus if the phage genome sequence is available, the *attB* site can usually be predicted by homology searching in the host chromosome. Interesting, these *attB* sites commonly overlap a host tRNA gene. In contrast, Int-S systems usually have shorter common cores (3-10 bp) and the *attB* sites cannot be readily predicted bioinformatically (*Kim* et al., 2003). Because of the overall diversity of the integration systems, at least 14 *attB* sites have been identified in *M. smegmatis* (*Hatfull*, 2022).

Some mycobacteriophages use an unusual system for lysogenic establishment referred to as 'integration-dependent immunity' (*Broussard* and *Hatfull*, 2013, *Broussard* et al., 2013) (Figure 3). The tell-tale feature of these is that the *attP* site is located within the repressor reading frame of the phage. This would seem counterintuitive, as integrative recombination will result in loss of the 3' part of the repressor gene, rendering it non-functional. In fact, the opposite is true. The viral form of the repressor carries a C-terminal SsrA-like tag that targets the protein for degradation by host proteases and is functionally incapable of conferring immunity to superinfection (*Broussard* et al., 2013). Integration results in loss of the degradation tag, stable protein is expressed, and immunity is established (Figure 3). Interestingly the Int-Y protein also carries a C-terminal degradation tag such that the stability of the integrase fundamentally determines the frequency of lysogenisation. Although these integration-dependent immunity systems were discovered in phages of *M. smegmatis*, they are quite prevalent among extant prophages of *Mycobacterium* genomes, especially those of *Mycobacterium abscessus* (*Dedrick* et al., 2021a).

MYCOBACTERIOPHAGE - HOST DYNAMICS

The determinants of phage host range and the dynamic interactions between bacteria and their phages are complex, and there is much to learn. *Mycobacterium* strains have been shown to carry restriction-modification systems (*Shankar* and *Tyagi*, 1993a, 1993b) and *M. tuberculosis* carries a CRISPR locus, although most mycobacterial strains are CRISPR-free (*He* et al.,

2012). Receptor variation is likely also important, although few mycobacteriophage receptors have been identified, other than the potential role of glycopeptidolipids in phage I3 infection (*Chen* et al., 2009). However, we note that a number of phage host range mutants have been reported with substitutions in tail spike proteins suggesting the direct role of phage-receptor interactions (*Jacobs-Sera* et al., 2012, *Dedrick* et al., 2019a, *Guerrero-Bustamante* et al., 2021). It is likely that there are a multitude of additional bacterial mechanisms influencing phage host ranges.

Prophage-mediated viral defence systems

Interestingly, temperate phages are directly involved in phage host range determination, because some prophages express defence genes that prevent productive infection of other unrelated phages (*Bondy-Denomy* et al., 2016). A multitude of such defence systems have been described in Cluster N phages (*Dedrick* et al., 2017a), but they are also present in other mycobacteriophages (*Gentile* et al., 2019). There are likely many yet to be discovered. Some of these may act by interference with DNA injection and viral exclusion similar to a related system in *E. coli* (*Cumby* et al., 2012, *Dedrick* et al., 2017a), whereas others appear to act after DNA injection has occurred and are mediated by abortive infection (*Dedrick* et al., 2017a). These defence systems are often quite specific for particular phages, and the specificities are unpredictable and need to be experimentally determined. In one notable case, an *M. smegmatis* lysogen carrying a Sbash prophage confers defence to infection by phage Crossroads (*Gentile* et al., 2019). Sbash (Subcluster I2) and Crossroads (Subcluster L2) are unrelated at the sequence level, and remarkably, the Sbash defence does not operate against any of the other eight Subcluster L2 phages tested, all of which are very closely related to Crossroads (*Gentile* et al., 2019). Discovery of such defence systems is only possible by making use of a very large collection of genomically-defined phages isolated on the same bacterial isolate (*M. smegmatis* mc^2155), and carefully determining their efficiencies of plating on a lysogenic strain relative to the non-lysogenic parent (*Gentile* et al., 2019).

Exclusion of superinfection in lytic growth

The competition in nature is not just between phages and their bacterial hosts, but also between lytically growing phages. When phages are growing lytically, there is strong selection for optimization of phage production, which can be threatened via superinfection by phage particles that seek to use microbial resources. It is thus no surprise that phages express genes in lytic growth that exclude superinfection by other phages (*Ko* and *Hatfull*, 2018). This can also be surprisingly specific and unpredictable bioinformatically. Experimentally identifying such functions, however, can also be challenging. One approach that has been informative is to use a surrogate assay that simply screens for phage genes that are toxic when expressed in the host (*Ko* and *Hatfull*, 2020). Some such genes act by interfering with the functions of essential host proteins that phages need for productive infection, and are thus toxic when expressed, although toxicity *per se* is irrelevant for phage growth. One good example is gp52 in phage Fruitloop (Subcluster F1). While gp52 is not required for Fruitloop lytic growth, it is expressed early in Fruitloop infection and interacts directly with the essential host

DivIVA (Wag31) protein (*Ko* and *Hatfull*, 2018). Fruitloop gp52 was discovered through a screen for proteins toxic to *M. smegmatis* and its interference with DivIVA specifically prevents infection by unrelated phage Rosebush (Subcluster B1).

A striking feature of phage genomes is the abundance of relatively small genes – leading to an average gene size that is only two-thirds of that of bacterial genes – most of which are of unknown function. What do all these genes do, and why are they present in phage genomes? Where they have been tested, many or most of these are expressed lytically but are not required for lytic growth (*Dedrick* et al., 2013). We suggest that most of these act to influence host-viral dynamics and to determine host range, by conferring defence against viral infection of lysogens, by excluding superinfection by other phages during lytic growth, or by acting in counter-defence against both host- and prophage-encoded defence systems. The specificity of these interactions complicates the untangling of this vast set of interactions, and thwarts simple bioinformatic or machine-learning strategies for predicting host range. But with many hundreds of thousands of phage genes of unknown function – just within the actinobacteriophage genomes – new approaches are needed to address these questions.

THERAPEUTIC POTENTIAL OF MYCOBACTERIOPHAGES

Many *Mycobacterium* pathogens cause diseases that are challenging to treat with antibiotics, including *M. tuberculosis* strains with multiple acquired resistances, and intrinsically resistant nontuberculous Mycobacterium (NTM) infections (*Nick* et al., 2021, *Mirzayev* et al., 2021). There is thus a need for new therapeutic approaches, and it is plausible that mycobacteriophages could contribute to the arsenal of anti-Mycobacterial 'drugs'. However, the opportunities and challenges are quite different for considering the therapeutic use of phages for NTM versus TB infections.

The first therapeutic use of mycobacteriophages

The first therapeutic use of mycobacteriophages was for a paediatric cystic fibrosis (CF) patient who had a disseminated *M. abscessus* infection following a bilateral lung transplant (*Dedrick* et al., 2019a). The infection could not be resolved with antibiotics, was life threatening, and the patient was at home on palliative care. The first challenge was to identify potentially useful phages, as few if any phages had been isolated on any *M. abscessus* strain and attempts at phage discovery using the specific patient strain (GD01) were not productive. A carefully chosen subset of *M. smegmatis* phages (based on the genomic relationships and host range information using *M. tuberculosis*) were then screened against the *M. abscessus* GD01 strain (*Dedrick* et al., 2019a). One lytic phage called Muddy infected GD01, while two others that infected (BPs and ZoeJ) were both temperate, and therefore needed to be engineered to inactivate the repressor gene (*Sampson* et al., 2009, *Dedrick* et al., 2019b). One of them (BPs) only infected *M. abscessus* GD01 at a reduced efficiency of plating, but a host range mutant (HRM) was isolated that efficiently infects the pathogenic strain. A three-phage cocktail was assembled, in the hope that these genomically diverse phages would infect through different

mechanisms such that resistance arising to one phage would not confer resistance to the others. The three phages were amplified, purified, and the cocktail was administered intravenously twice daily at a dose of 10^9 plaque forming units (PFU)/ml. The phages were provided as an adjunct to the ongoing antibiotic regimen. The patient showed substantial improvement, with resolution of an infected node in the liver, healing of the infected sternal transplant wound, and clearance of skin nodules (*Dedrick* et al., 2019a).

Broadening the therapeutic use of mycobacteriophages

Can the therapeutic use of mycobacteriophages be extended to other patients with NTM infections? Addressing this has been facilitated by screening a large series (~250) of clinical isolates for phage susceptibility, learning about these profiles, and providing phages for additional compassionate-use cases where possible (*Dedrick* et al., 2021b, 2022). A key finding is that a substantial proportion (~40%) of the *M. abscessus* isolates have a smooth colony morphotype and no phages have been identified that efficiently infect *and* kill any of these strains. The other ~60% have a rough colony morphotype strains, with at least one potentially therapeutic phage identified for about 75% of these strains. This raises several additional questions. First, because the total number of potentially therapeutically useful different phages that have been identified is only around six to eight, where will additional phages come from? Second, how can we extend the proportion of rough strains that can be treated? Third, if strains are only sensitive to a single phage, can that phage be used without failure due to phage resistance? Fourth, how will phages be discovered with utility for smooth colony strains? Some preliminary answers are emerging for at least some of these questions.

In terms of identifying additional phages that could be used therapeutically, it is helpful to recognize that what is needed are phages that are genomically different to those already available. Such phages could be used in cocktails with extant phages to try and avoid resistance, or may have new tropisms (host preferences) that expand the proportion of rough strains that can be infected and killed. Several approaches to identifying more of these phages are plausible. First, although few phages have been identified using *M. abscessus* as a host for isolation, this warrants substantial additional effort, using a wider range of strains. Second, the current and new phages isolated on *M. smegmatis* may continue to be a fruitful source, especially through the isolation of HRMs that have acquired the ability to efficiently infect one or more of the *M. abscessus* isolates (*Dedrick* et al., 2021b). Finally, there is also the prospect of exploiting the prophages integrated into *M. abscessus* genomes (*Dedrick* et al., 2021a). Many of these appear to be intact and capable of lytic growth, and spontaneously induced particles are present in culture supernatants. The challenge then is to find another *M. abscessus* strain on which these phages can grow lytically and form plaques. An attractive feature of this approach is that ~85% of *M. abscessus* clinical isolates have one or more prophages integrated into their genomes, and some have as many as six prophages (*Dedrick* et al., 2021b). So, there is a wealth of such prophages which constitutes a large resource that can be exploited, and bioinformatic analyses show that these are distinct from the *M. smegmatis* phages (*Dedrick* et al., 2021a, 2021b). Pairwise screening has facilitated lytic growth of

Table 1: Summary of compassionate-use cases of phage treatment of Mycobacterium infections

Patient #	Strain	# phages	Resistance?[1]	Route[2]	Neut?[3]	Outcomes
1	*M. abscessus*	3	No	IV	Y/N/N	Favorable resolution for ~3 years
2	*M. abscessus*	1	No	IV	NT	Conversion to smear-negative
3	*M. abscessus*	2	NT	IV	N	Deceased, organ failure; not phage-related
4	*M. abscessus*	1	NT	IV	NT	Conversion to smear-negative
5	*M. abscessus*	1	No	IV	N	No substantial clinical improvement
6	*M. abscessus*	1	NT	IV	NT	No substantial clinical improvement
7	*M. abscessus*	1	No	IV	N	Conversion to culture negative (rough strain)
8	*M. abscessus*	1	No	IV/Neb	Y	Intermittently smear negative
9	*M. abscessus*	2	NT	IV	N	Favorable resolution
10	*M. abscessus*	2	NT	IV	NT	Conversion to culture/smear negative
11	*M. abscessus*	1	No	IV	Y	Intermittently smear negative
12	*M. abscessus*	3	Partial to one	IV/Neb	Y	Transient improvement only
13	*M. abscessus*	1	No	IV/Neb	N	FEV1 improvement; culture positive
14	*M. abscessus*	2	NT	IV	NT	No substantial clinical improvement
15	*M. abscessus*	2	No	IV	Y	Conversion to culture negative; transplant
16	*M. chelonae*	1	NT	IV	Y	Favorable resolution; negative biopsies
17	*M. avium*	1	NT	IV/Neb	N	FEV1 improvement; culture negative
18	*M. abscessus*	1	No	IV/Neb	Y	Ongoing
19	*M. abscessus*	2	No	IV/Neb	Y	No substantial clinical improvement
20	BCG	3	NT	IV	N	Improved clinically

[1]Resistance was determined by screening bacterial isolates recovered following the start of phage treatment. NT, not tested.
[2]The routes of administration were intravenous (IV) or nebulized (neb), or IV followed subsequently by additional of nebulization (IV/neb).
[3]Neutralization was determined *in vitro*. NT, not tested.

spontaneously induced prophages, and these can then be engineered to prevent lysogeny and potentially added to the therapeutic arsenal. These potential paths towards the expansion of the therapeutic phage bank should expand the number of rough strain morphotype infections that can be treated.

In the first treatment case, a cocktail of three phages was used because of concerns about phage resistance. However, subsequent observations have shown that phage resistance occurs relatively infrequently in *M. abscessus* strains, and resistance has not been encountered when used clinically, even in treatments where only a single phage was deployed (*Dedrick* et al., 2022, 2021b). Some phage-resistant *M. abscessus* mutants have been recovered *in vitro*, but it is not yet clear if these have compromised fitness for *in vivo* growth in the patient (*Dedrick* et al., 2021b).

A consecutive series of 20 compassionate use phage therapies for Mycobacterium infections

To date, a total of 20 case studies have been reported, a majority of which (11/20) show favourable microbiological or clinical indications (*Dedrick* et al., 2022) (Table 1). However, in four cases there was no evident clinical improvement, and in the remaining cases, treatment is either ongoing or incomplete. No adverse reactions were observed, and in several cases strongly favourable outcomes are reported, including resolution of a pulmonary infection in a CF patient (*Nick* et al., 2022), and resolution of a disseminated *Mycobacterium chelonae* cutaneous infection in a mildly immunosuppressed patient (*Little* et al., 2022). Together, these cases provide considerable encouragement for continued exploration of phage treatments for NTM infections, highlighting some opportunities and some limitations. For example, in 11 cases, only a single phage was used because it was the only phage to which the strain was sensitive. In these cases, however, phage resistance was not observed in any case nor was it the cause of treatment failure. Although this was unexpected, it bodes well for future treatments, moderating one of the major concerns in the general therapeutic use of phages (*Dedrick* et al., 2022) (Table 1). It is also noteworthy that these treatments are lengthier (years in some cases) than in phage therapies for other diseases, and these are not trivial interventions. But we know little about optimal dosage, routes of administration, or phage pharmacokinetics, and blinded clinical trials are needed to address these unknowns. Nonetheless, these case studies provide insights into safety and clinical and microbiological parameters to monitor during treatments that are invaluable for trial design and effective implementation (*Dedrick* et al., 2022, *Nick* et al., 2022).

Interestingly, clinical isolates of *M. tuberculosis* have limited genetic diversity and relatively minor variations in phage infections profiles (*Guerrero-Bustamante* et al., 2021). A set of five distinct phages, taking advantage of engineering and isolation of HRMs – as described above for NTMs – constitute a potential cocktail for a broad range of clinical isolates, although only some of these five might ultimately be needed. And subsets of these could be administered sequentially to negate impediments presented by neutralization. However, compassionate use cases of TB that are both suitable for phage treatment and meet regulatory requirements are rare, and initial clinical trials would seem warranted.

SUMMATION

The study of mycobacteriophages has contributed major advances to our understanding of phage diversity, phage biology including expression and regulation, host-virus dynamics, science education, and clinical utility for treating *Mycobacterium* diseases. However, there is still much to learn, to explore, and to discover. Many of the phages have not been investigated beyond genomic characterization and will give new insights when studied in detail. Collectively, there are hundreds of thousands of genes of unknown function and elucidating their roles will require new and higher-throughput strategies. We also know little about host range beyond the inclusion of infection profiles for the pathogens *M. tuberculosis* and *M. abscessus*. Our current understanding of mycobacteriophages is largely based on those isolated using *M. smegmatis* mc^2155 as a host, and it is likely that new and different types of phages would be discovered when using other mycobacterium hosts. We note that this is challenging for very slow growing strains and pathogens, but insights from prophages of sequenced NTM strains suggests there's an abundance of diverse phages awaiting discovery, many of which will add to the arsenal of therapeutic options.

ACKNOWLEDGEMENTS

I am extremely grateful to all members of my research group that have contributed to our understanding of mycobacteriophages over a span of many years. Many thanks to Bekah Dedrick, Dan Russell, and Debbie Jacobs-Sea for comments on the manuscript. This work was supported by funding from National Institutes of Health grant GM131729.

LITERATURE

Bernheim, A. and Sorek, R.: The pan-immune system of bacteria: antiviral defence as a community resource. Nat. Rev. Microbiol. 18, 113-119 (2020).

Bondy-Denomy, J., Qian, J., Westra, E.R., Buckling, A., Guttman, D.S., Davidson, A.R., and Maxwell, K.L.: Prophages mediate defense against phage infection through diverse mechanisms. ISME J. 10, 2854-2866 (2016).

Broussard, G.W. and Hatfull, G.F.: Evolution of genetic switch complexity. Bacteriophage 3, e24186 (2013).

Broussard, G.W., Oldfield, L.M., Villanueva, V.M., Lunt, B.L., Shine, E.E., and Hatfull, G.F.: Integration-dependent bacteriophage immunity provides insights into the evolution of genetic switches. Mol. Cell. 49, 237-248 (2013).

Brown, K.L., Sarkis, G.J., Wadsworth, C., and Hatfull, G.F.: Transcriptional silencing by the mycobacteriophage L5 repressor. EMBO J. 16, 5914-5921 (1997).

Cater, J.C. and Redmond, W.B.: Isolation of and studies on bacteriophage active against mycobacteria. Can. J. Microbiol. 7, 697-703.(1961).

Chen, J., Kriakov, J., Singh, A., Jacobs, W.R., Besra, G.S., and Bhatt, A.: Defects in glycopeptidolipid biosynthesis confer phage I3 resistance in Mycobacterium smegmatis. Microbiology 155, 4050-4057 (2009).

Cumby, N., Edwards, A.M., Davidson, A.R., and Maxwell, K.L.: The bacteriophage

HK97 gp15 moron element encodes a novel superinfection exclusion protein. J. Bacteriol. 194, 5012-5019 (2012).

Dedrick, R.M., Marinelli, L.J., Newton, G.L., Pogliano, K., Pogliano, J., and Hatfull, G.F.: Functional requirements for bacteriophage growth: gene essentiality and expression in mycobacteriophage Giles. Mol. Microbiol. 88: 577-589 (2013).

Dedrick, R.M., Jacobs-Sera, D., Bustamante, C.A., Garlena, R.A., Mavrich, T.N., Pope, W.H., Reyes, J.C., Russell, D.A., Adair, T., Alvey, R., Bonilla, J.A., Bricker, J.S., Brown, B.R., Byrnes, D., Cresawn, S.G., Davis, W.B., Dickson, L.A., Edgington, N.P., Findley, A.M., Golebiewska, U., Grose, J.H., Hayes, C.F., Hughes, L.E., Hutchison, K.W., Isern, S., Johnson, A.A., Kenna, M.A., Klyczek, K.K., Mageeney, C.M., Michael, S.F., Molloy, S.D., Montgomery, M.T., Neitzel, J., Page, S.T., Pizzorno, M.C., Poxleitner, M.K., Rinehart, C.A., Robinson, C.J., Rubin, M.R., Teyim, J.N., Vazquez, E., Ware, V.C., Washington, J., and Hatfull, G.F.: Prophage-mediated defence against viral attack and viral counter-defence. Nat. Microbiol. 2, 16251 (2017a).

Dedrick, R.M., Mavrich, T.N., Ng, W.L., and Hatfull, G.F.: Expression and evolutionary patterns of mycobacteriophage D29 and its temperate close relatives. BMC Microbiol. 17, 225 (2017b).

Dedrick, R., Guerrero Bustamante, C., Garlena, R.A., Russell, D.A., Ford, K., Harris, K., Gilmour, K.C., Soothill, J., Jacobs-Sera, D., Schooley, R.T., Hatfull, G.F., and Spencer, H.: Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant Mycobacterium abscessus. Nat. Med. 25, 730-733 (2019a).

Dedrick, R.M., Guerrero Bustamante, C.A., Garlena, R.A., Pinches, R.S., Cornely, K., and Hatfull, G.F.: Mycobacteriophage ZoeJ: A broad host-range close relative of mycobacteriophage TM4. Tuberculosis (Edinb.) 115, 14-23 (2019b).

Dedrick, R.M., Aull, H.G., Jacobs-Sera, D., Garlena, R.A., Russell, D.A., Smith, B.E., Mahalingam, V., Abad, L., Gauthier, C.H., and Hatfull, G.F.: The Prophage and Plasmid Mobilome as a Likely Driver of Mycobacterium abscessus Diversity. mBio. 12, e03441-20 (2021a).

Dedrick, R.M., Smith, B.E., Garlena, R.A., Russell, D.A., Aull, H.G., Mahalingam, V., Divens, A.M., Guerrero-Bustamante, C.A., Zack, K.M., Abad, L., Gauthier, C.H., Jacobs-Sera, D., and Hatfull, G.F.: Mycobacterium abscessus Strain Morphotype Determines Phage Susceptibility, the Repertoire of Therapeutically Useful Phages, and Phage Resistance. mBio. 12, e03431-20 (2021b).

Dedrick, R.M., B.E. Smith, M. Cristinziano, K.G. Freeman, D. Jacobs-Sera, Y. Belessis, A.W. Brown, K.A. Cohen, R.M. Davidson, D. van Duin, A. Gainey, C.B. Garcia, C.R.B. George, G. Haidar, W. Ip, J. Iredell, A. Khatami, J.S. Little, K. Malmivaara, B.J. McMullan, D.E. Michalik, A. Moscatelli, J.A. Nick, M.G. Tupayachi Ortiz, H.M. Polenakovik, P.D. Robinson, M. Skurnik, D.A. Solomon, J. Soothill, H. Spencer, P. Wark, A. Worth, R.T. Schooley, C.A. Benson, and G.F. Hatfull, (2022) Phage Therapy of Mycobacterium Infections: Compassionate-use of Phages in Twenty Patients with Drug-Resistant Mycobacterial Disease. Clin. Infect. Dis., ciac453, Epub ahead of print (2022).

Doke, S.: Studies on mycobacteriophages and lysogenic mycobacteria. J. Kumamoto Med. Soc. 34, 1360-1373 (1960).

Donnelly-Wu, M.K., Jacobs, Jr., W.R., and Hatfull, G.F.: Superinfection immunity of mycobacteriophage L5: applications for genetic transformation of mycobacteria. Mol. Microbiol. 7, 407-417 (1993).

Ford, M.E., Sarkis, G.J., Belanger, A.E., Hendrix, R.W., and Hatfull, G.F.: Genome structure of mycobacteriophage D29: implications for phage evolution. J. Mol. Biol. 279, 143-164 (1998).

Gentile, G.M., Wetzel, K.S., Dedrick, R.M., Montgomery, M.T., Garlena, R.A., Jacobs-Sera, D., and Hatfull, G.F.: More Evidence of Collusion: a New Prophage-Mediated Viral Defense System Encoded by

Mycobacteriophage Sbash. mBio. 10, e00196-19 (2019).
Good, R.C. and Mastro, T.D.: The modern mycobacteriology laboratory. How it can help the clinician. Clin. Chest Med. 10, 315-322 (1989).
Guerrero-Bustamante, C.A., Dedrick, R.M., Garlena, R.A., Russell, D.A., and Hatfull, G.F.: Toward a Phage Cocktail for Tuberculosis: Susceptibility and Tuberculocidal Action of Mycobacteriophages against Diverse Mycobacterium tuberculosis Strains. mBio 12, e00973-21 (2021).
Hampton, H.G., Watson, B.N.J., and Fineran, P.C.: The arms race between bacteria and their phage foes. Nature 577, 327-336 (2020).
Hanauer, D.I., Graham, M.J., Sea, P., Betancur, L., Bobrownicki, A., Cresawn, S.G., Garlena, R.A., Jacobs-Sera, D., Kaufmann, N., Pope, W.H., Russell, D.A., Jacobs, Jr., W.R., Sivanathan, V., Asai, D.J., and Hatfull, G.F.: An inclusive Research Education Community (iREC): Impact of the SEA-PHAGES program on research outcomes and student learning. Proc. Natl. Acad. Sci. USA 114, 13531-13536 (2017).
Hanauer, D.I., Jacobs-Sera, D., Pedulla, M.L., Cresawn, S.G., Hendrix, R.W., and Hatfull, G.F: Inquiry learning. Teaching scientific inquiry. Science 314, 1880-1881 (2006).
Hatfull, G.F.: Mycobacteriophages. In: The Bacteriophages (Ed.: Calendar, R.). Oxford University Press, New York, NY, 602-620 (2006).
Hatfull, G.F.: Dark Matter of the Biosphere: the Amazing World of Bacteriophage Diversity. J. Virol. 89, 8107-8110 (2015a).
Hatfull, G.F.: Innovations in Undergraduate Science Education: Going Viral. J Virol. 89, 8111-8113 (2015b).
Hatfull, G.F.: Actinobacteriophages: Genomics, Dynamics, and Applications. Annu. Rev. Virol. 7, 37-61 (2020).
Hatfull, G.F.: Mycobacteriophages: From Petri Dish to Patient. PLoS Pathog. 18, e1010602 (2022).
Hatfull, G.F. and Sarkis, G.J.: DNA sequence, structure and gene expression of mycobacteriophage L5: a phage system for mycobacterial genetics. Mol. Microbiol. 7, 395-405 (1993).
Hatfull, G.F. and Hendrix, R.W.: Bacteriophages and their Genomes. Curr. Opin. Virol. 1, 298-303 (2011).
Hatfull, G.F., Pedulla, M.L., Jacobs-Sera, D., Cichon, P.M., Foley, A., Ford, M.E., Gonda, R.M., Houtz, J.M., Hryckowian, A.J., Kelchner, V.A., Namburi, S., Pajcini, K.V., Popovich, M.G., Schleicher, D.T., Simanek, B.Z., Smith, A.L., Zdanowicz, G.M., Kumar, V., Peebles, C.L., Jacobs, Jr., W.R., Lawrence, J.G., and Hendrix, R.W.: Exploring the mycobacteriophage metaproteome: phage genomics as an educational platform. PLoS Genet. 2, e92 (2006).
Hatfull, G.F., Jacobs-Sera, D., Lawrence, J.G., Pope, W.H., Russell, D.A., Ko, C.C., Weber, R.J., Patel, M.C., Germane, K.L., Edgar, R.H., Hoyte, N.N., Bowman, C.A., Tantoco, A.T., Paladin, E.C., Myers, M.S., Smith, A.L., Grace, M.S., Pham, T.T., O'Brien, M.B., Vogelsberger, A.M., Hryckowian, A.J., Wynalek, J.L., Donis-Keller, H., Bogel, M.W., Peebles, C.L., Cresawn, S.G., and Hendrix, R.W.: Comparative Genomic Analysis of 60 Mycobacteriophage Genomes: Genome Clustering, Gene Acquisition, and Gene Size. J. Mol. Biol. 397, 119-143 (2010).
He, L., Fan, X., and Xie, J.: Comparative genomic structures of Mycobacterium CRISPR-Cas. J. Cell. Biochem. 113, 2464-2473 (2012).
Hendrix, R.W.: Bacteriophages: evolution of the majority. Theor. Popul. Biol. 61, 471-480 (2002).
Hendrix, R.W., Smith, M.C., Burns, R.N., Ford, M.E., and Hatfull, G.F.: Evolutionary relationships among diverse bacteriophages and prophages: all the world's a phage. Proc. Natl. Acad. Sci. USA 96, 2192-2197 (1999).
Huson, D.H.: SplitsTree: analyzing and visualizing evolutionary data. Bioinformatics 14, 68-73 (1998).
Jacobs, Jr., W.R.: *Mycobacterium tuberculosis*: A Once Genetically Intractable Organism.

In: Molecular Genetics of the Mycobacteria. (Eds.: Hatfull, G.F. and Jacobs Jr., W.R.). ASM Press, Washington, DC, 1-16 (2000).

Jacobs-Sera, D., Marinelli, L.J., Bowman, C., Broussard, G.W., Guerrero Bustamante, C., Boyle, M.M., Petrova, Z.O., Dedrick, R.M., Pope, W.H., Science Education Alliance Phage Hunters Advancing Genomics And Evolutionary Science Sea-Phages Program; Modlin, R.L., Hendrix, R.W., and Hatfull, G.F.: On the nature of mycobacteriophage diversity and host preference. Virology 434, 187-201 (2012).

Jordan, T.C., Burnett, S.H., Carson, S., Caruso, S.M., Clase, K., DeJong, R.J., Dennehy, J.J., Denver, D.R. , Dunbar, D., Elgin, S.C., Findley, A.M., Gissendanner, C.R., Golebiewska, U.P., Guild, N., Hartzog, G.A., Grillo, W.H., Hollowell, G.P., Hughes, L.E., Johnson, A., King, R.A., Lewis, L.O., Li, W., Rosenzweig, F., Rubin, M.R., Saha, M.S., Sandoz, J., Shaffer, C.D., Taylor, B., Temple, L., Vazquez, E., Ware, V.C., Barker, L.P., Bradley, K.W., Jacobs-Sera, D., Pope, W.H., Russell, D.A., Cresawn, S.G., Lopatto, D., Bailey C.P., and Hatfull, G.F.: A broadly implementable research course in phage discovery and genomics for first-year undergraduate students. mBio. 5, e01051-13 (2014).

Kim, A.I., Ghosh, P., Aaron, M.A., Bibb, L.A., Jain, S., and Hatfull, G.F.: Mycobacteriophage Bxb1 integrates into the Mycobacterium smegmatis groEL1 gene. Mol. Microbiol. 50, 463-473 (2003).

Ko, C.C. and Hatfull, G.F.: Mycobacteriophage Fruitloop gp52 inactivates Wag31 (DivIVA) to prevent heterotypic superinfection. Mol. Microbiol. 108, 443-460 (2018).

Ko, C.C. and Hatfull, G.F.: Identification of mycobacteriophage toxic genes reveals new features of mycobacterial physiology and morphology. Sci. Rep. 10, 14670 (2020).

Lee, M.H., Pascopella, L., Jacobs, Jr., W.R., and Hatfull, G.F.: Site-specific integration of mycobacteriophage L5: integration-proficient vectors for Mycobacterium smegmatis, Mycobacterium tuberculosis, and bacille Calmette-Guerin. Proc. Natl. Acad. Sci. USA 88, 3111-3115 (1991).

Lewis, J.A. and Hatfull, G.F.: Control of directionality in integrase-mediated recombination: examination of recombination directionality factors (RDFs) including Xis and Cox proteins. Nucleic Acids Res. 29, 2205-2216 (2001).

Little, J.S., Dedrick, R.M., Freeman, K.G., Cristinziano, M., Smith, B.E., Benson, C.A., Jhaveri, T.A., Baden, L.R., Solomon, D.A., and Hatfull, G.F.: Bacteriophage treatment of disseminated cutaneous Mycobacterium chelonae infection. Nat. Commun. 13, 2313 (2022).

Mavrich, T.N. and Hatfull, G.F.: Bacteriophage evolution differs by host, lifestyle and genome. Nat. Microbiol. 2, 17112 (2017).

Mirzayev, F., Viney, K., Linh, N.N., Gonzalez-Angulo, L., Gegia, M., Jaramillo, E., Zignol, M. and Kasaeva, T.: World Health Organization recommendations on the treatment of drug-resistant tuberculosis, 2020 update. Eur. Respir. J. 57, 2003300 (2021).

Mizuguchi, Y.: Mycobacteriophages. In: The Mycobacteria: A Sourcebook (Eds.: Kubica, G.P. and Wayne, L.G.). Marcel Dekker, Inc., New York, 641-662 (1984).

Murohashi, T., Tokunaga, T., Mizuguchi, Y., and Maruyama, Y.: Phage Typing of Slow-Growing Mycobacteria. Am. Rev. Respir. Dis. 88, 664-669 (1963).

Nesbit, C.E., Levin, M.E., Donnelly-Wu, M.K., and Hatfull, G.F.: Transcriptional regulation of repressor synthesis in mycobacteriophage L5. Mol. Microbiol. 17, 1045-1056 (1995).

Nick, J.A., Daley, C.L., Lenhart-Pendergrass, P.M., and Davidson, R.M.: Nontuberculous mycobacteria in cystic fibrosis. Curr. Opin. Pulm. Med. 27, 586-592 (2021).

Nick, J.A., Dedrick, R.M., Gray, A.L., Vladar, E.K., Smith, B.E., Freeman, K.G., Malcolm, K.C., Epperson, L.E., Hasan, N.A., Hendrix, J., Callahan, K., Walton, K., Vestal, B., Wheeler, E., Rysavy, N.M., Poch, K., Caceres, S., Lovell, V.K., Hisert,

K.B., de Moura, V.C., Chatterjee, D., De, P., Weakly, N., Martiniano, S.L., Lynch, D.A., Daley, C.L., Strong, M., Jia, F., Hatfull, G.F., and Davidson, R.M.: Host and pathogen response to bacteriophage engineered against Mycobacterium abscessus lung infection. Cell 185, 1860-1874 (2022).

Payne, K., Sun, Q., Sacchettini, J., and Hatfull, G.F.: Mycobacteriophage Lysin B is a novel mycolylarabinogalactan esterase. Mol. Microbiol. 73, 367-381 (2009).

Payne, K.M. and Hatfull, G.F.: Mycobacteriophage endolysins: diverse and modular enzymes with multiple catalytic activities. PLoS One 7, e34052 (2012).

Pedulla, M.L., Ford, M.E., Houtz, J.M., Karthikeyan, T., Wadsworth, C., Lewis, J.A., Jacobs-Sera, D., Falbo, J., Gross, J., Pannunzio, N.R., Brucker, W., Kumar, V., Kandasamy, J., Keenan, L., Bardarov, S., Kriakov, J., Lawrence, J.G., Jacobs, W.R., Hendrix, R.W., and Hatfull, G.F.: Origins of highly mosaic mycobacteriophage genomes. Cell 113: 171-182 (2003).

Pope, W.H., Bowman, C.A., Russell, D.A., Jacobs-Sera, D., Asai, D.J., Cresawn, S.G., Jacobs, W.R., Hendrix, R.W., Lawrence, J.G., Hatfull, G.F., Science Education Alliance Phage Hunters Advancing Genomics and Evolutionary Science; Phage Hunters Integrating Research and Education; and Mycobacterial Genetics Course: Whole genome comparison of a large collection of mycobacteriophages reveals a continuum of phage genetic diversity. Elife 4, e06416 (2015).

Pope, W.H., Mavrich, T.N., Garlena, R.A., Guerrero-Bustamante, C.A., Jacobs-Sera, D., Montgomery, M.T., Russell, D.A., Warner, M.H., Science Education Alliance-Phage Hunters Advancing Genomics and Evolutionary Science (SEA-PHAGES); and Hatfull, G.F.: Bacteriophages of *Gordonia* spp. Display a Spectrum of Diversity and Genetic Relationships. mBio. 8, e01069-17 (2017).

Ptashne, M.: A Genetic Switch. Blackwel Science Ltd. and Cell Press, Oxford/Cambridge (1987).

Sampson, T., Broussard, G.W., Marinelli, L.J., Jacobs-Sera, D., Ray, M., Ko, C.C., Russell, D., Hendrix, R.W., and Hatfull, G.F.: Mycobacteriophages BPs, Angel and Halo: comparative genomics reveals a novel class of ultra-small mobile genetic elements. Microbiology 155, 2962-2977 (2009).

Shankar, S. and Tyagi, A.K.: MchAI and MchAII, two class-II restriction endonucleases from Mycobacterium chelonei. Gene 132, 119-123 (1993a).

Shankar, S. and Tyagi, A.K.: Purification and characterization of restriction endonuclease MgoI from Mycobacterium gordonae. Gene 131, 153-154 (1993b).

Snider, Jr., D.E., Jones, W.D., and Good, R.C.: The usefulness of phage typing Mycobacterium tuberculosis isolates. Am. Rev. Respir. Dis. 130, 1095-1099 (1984).

Takeya, K., Koike, M., Mori, R., and Toda, T.: Light and electron microscope studies of mycobacterium--mycobacteriophage interactions. III. Further studies on the ultrathin sections. J. Biophys. Biochem. Cytol. 11, 441-447 (1961).

Takeya, K., Koike, M., Mori, R., Yuda, Y., and Toda, T.: Light and electron microscope studies of Mycobacterium-mycobacteriophage interactions. II. Electron microscope studies. J. Bacteriol. 78, 313-319 (1959a).

Takeya, K., Mori, R., Nakashima, N., Koike, M., and Toda, T.: Light and electron microscope studies of Mycobacterium-mycobacteriophage interactions. I. Light microscope studies. J. Bacteriol. 78, 307-312 (1959b).

Wetzel, K.S., Aull, H.G., Zack, K.M., Garlena, R.A., and Hatfull, G.F.: Protein-Mediated and RNA-Based Origins of Replication of Extrachromosomal Mycobacterial Prophages. mBio. 11, e00385-20 (2020).

PF BACTERIOPHAGE INHIBIT NEUTROPHIL MIGRATION IN THE LUNG

MEDEEA C. POPESCU[1,2], NINA PENNETZDORFER[2], AVIV HARGIL[2], and PAUL L. BOLLYKY[1,2]

[1]Immunology Program, Stanford University, and [2]Division of Infectious Diseases, Stanford University, Stanford, CA 94305, USA

SUMMARY

Bacteriophage are abundant in the human body, including at sites of infection. We report that Pf4 phage, a filamentous bacteriophage produced by *Pseudomonas aeruginosa*, dampens inflammatory responses in response to either *P. aeruginosa* airway infection in a mouse model of acute pneumonia or bacterial endotoxin treatment. Pf4 triggers TLR3-dependent type I interferon production and antagonize production of anti-bacterial cytokines and chemokines. In particular, Pf4 phages inhibit CXCL5, preventing efficient neutrophil chemotaxis in response to endotoxin. These results suggest that Pf4 phages alter innate immunity to bacteria potentially dampening inflammation and neutrophil migration at sites of bacterial colonization or infection.

INTRODUCTION

Bacteriophages, viruses that parasitize bacteria, are abundant at sites of bacterial colonization and infection, including in the lungs of patients infected with *Pseudomonas aeruginosa*. However, it remains unclear how the innate immune system senses phages or how phages impact sensing of bacteria (*Popescu* et al., 2021). While some phages are potent immunogens and have been used for vaccine development (*Dąbrowska* et al., 2014; *de Vries* et al., 2021), other phages induce minimal inflammation, including phages used in phage therapy (*Cano* et al., 2021; *Liu* et al., 2021). The mechanisms that drive these distinctions are unclear.

We and others have previously investigated the impact of Pf phages on their bacterial and human hosts. Pf is a genus of filamentous bacteriophages that infect the common bacterial pathogen *P. aeruginosa*, and includes the phages Pf1-Pf8 (*Knezevic* et al., 2015; *Mai-Prochnow* et al., 2015; *Hay* and *Lithgow*, 2019; *Fiedoruk* et al., 2020). Pf phages are Inoviruses and have a single stranded (ss) DNA genome packaged within a helical filamentous structure made up of thousands of copies of the major coat protein CoaB (*Marvin* et al., 2014; *Hay* and *Lithgow*, 2019; *Roux* et al., 2019). Pf virions are ~ 6-7 nm in diameter and ~ 1-2 μm in length (*Janmey* et al., 2014). Approximately half of *P. aeruginosa* isolates harbour Pf phages (*Knezevic* et al., 2015; *Burgener* et al., 2019). Unlike lytic or lysogenic phages that lyse their bacterial hosts after replication, Pf phages follow a chronic infection life cycle whereby Pf virions are continuously extruded from the bacterial cell envelope without lysis (*Secor* et al., 2020).

Production of Pf4, a well-studied member of the Pf family, contributes to

bacterial phenotypes associated with *P. aeruginosa* chronic infection, including reduced twitching motility, increased adhesion, enhanced biofilm formation, and antibiotic tolerance (*Rice* et al., 2009; *Secor* et al., 2015, 2017; *Sweere* et al., 2019; *Tarafder* et al., 2020). Indeed, filamentous phages like Pf4 contribute to the fitness of their bacterial hosts and the pathogenesis of *P. aeruginosa* infections (*Mai-Prochnow* et al., 2015; *Bille* et al., 2017; *Pant* et al., 2020; *Pourtois* et al., 2021; *Schmidt* et al., 2022). Consistent with this, we previously reported that Pf phages are common in individuals with cystic fibrosis (CF) and that *P. aeruginosa* infections associated with Pf+ strains are characterized by older age, advanced lung disease, worse disease exacerbations, and antibiotic resistance in this group (*Burgener* et al., 2019). Pf phages are likewise associated with chronic *P. aeruginosa* wound infections (*Sweere* et al., 2019).

Along with effects on bacterial pathogenesis, Pf4 phage enables *P. aeruginosa* to evade the mammalian host immune response. In a model of acute lung infection, mice inoculated with *P. aeruginosa* supplemented with Pf4 phage did not develop sepsis, had less pulmonary inflammation, and survived significantly longer than mice infected with WT *P. aeruginosa* only (*Secor* et al., 2017). In a chronic wound infection model, Pf4 phage was associated with impaired phagocytosis and reduced levels of tumour necrosis factor alpha (TNF-α) (*Sweere* et al., 2019). These effects were mediated by Toll-like receptor 3 (TLR3) and type I interferon (IFN) production (*Sweere* et al., 2019). However, the nature of these responses was unclear given that Pf4 phage is a ssDNA virus and TLR3 recognizes double-stranded (ds) RNAs.

Here, we identify a role for Pf4 phage in inhibiting neutrophil chemotaxis in response to *P. aeruginosa* infection. We find that Pf4 phage downregulates the expression of neutrophil chemoattractants by human macrophages, resulting in impaired ability to recruit cells to the site of infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in the "Key Resources Table". The *P. aeruginosa* isolate mPAO1 served as a wildtype that harbours the filamentous phage Pf4 in all experiments (*Rice* et al., 2009). Isogenic phage-free strain PAO1ΔPf4 was derived from strain PAO1 that lacks Pf4 entirely but can be reinfected by Pf4 (*Rice* et al., 2009). Unless stated otherwise, all *P. aeruginosa* strains were grown with aeration in Brain Heart Infusion (BHI, BD Bacto™, Cat. No. 237200), or Luria-Bertani (LB Miller Broth, BD Bacto™, Cat. No. DF0446-07-5) at 37°C. *Escherichia coli* DH5α was used for plasmid maintenance and was grown in LB with aeration at 37°C. Antibiotics were used in the following final concentrations: gentamycin (10 μg/ml for plasmid maintenance in *E. coli*; 100 μg/ml for plasmid maintenance in *P. aeruginosa*, VWR, Cat. No. 97062-974).

Preparation and quantification of bacteriophages

P. aeruginosa mPAO1 was used to produce Pf4. Mock phage preparations from PAO1ΔPf4 were prepared according to the same protocol as other phage samples. Cultures were inoculated with

an OD_{600} of 0.1 and grown to the midlog phase in which the respective phage was added and co-incubated overnight. The next day, the bacteria were harvested at 6,000 x *g*; 30 minutes; 4°C. The supernatant was sterile filtered using a 0.22 µm bottle top filter and treated with 50 U/ml of benzonase nuclease (Sigma Aldrich, Cat. No. E1014-25KU) for 2 hours. The phage solution was incubated with polyethylene glycol 8000 (PEG-8000, Sigma-Aldrich, Cat. No. P2139-500G) at 4°C overnight as described previously (*Boulanger* et al., 2009) and dialyzed against PBS for 48 hours. Purified phage preparations were quantified by plaque assays as well as by qRT-PCR using primers directed against the major coat protein of the respective phage and a vector based (pBS-SK back bone) standard comprising the gene encoding for the respective major coat protein. Phages were stored in 1x PBS at 4°C. Endotoxin was quantified by EndoZyme II assay (BioVendor, Cat. No. 890030) as per manufacturer's directions.

Tissue culture

Human U937 macrophages (ATCC, Cat. No. CRL-1593.2™) were maintained in RPMI (Thermo Fisher Scientific, Cat. No. 11875093) supplemented with 10% FBS (RMBIO, Cat. No. FGR-BBT), penicillin-streptomycin (Fisher Scientific, Cat. No. MT30002CI) and sodium pyruvate (Thermo Fisher Scientific, Cat. No. 11360070).

Cell stimulation

For cytokine release and reporter assays, cells were seeded at a density of $5x10^4$ cells/well in a 96-well plate. Unless otherwise specified, stimuli were as follows: $1x10^8$ pfu/well of purified phage preparation and 100 ng/ml LPS (Invivogen, Cat. No. tlrl-eblps). The TLR3 signalling inhibitor (EMD Millipore, Cat. No. 614310) was used at 27 µM and anti-human IFNAR2 antibody (PBL Assay Science, Cat. No. 21385-1) was used at 100 ng/ml. Cells were incubated at 37ºC and 5% CO_2 for the indicated timepoints, then centrifuged at 300 x *g* for 5 minutes prior to supernatant removal.

Enzyme-linked immunosorbent assay (ELISA)

Human CXCL5 (BioLegend; Cat. No. 440904), human TNF-α (BioLegend; Cat. No. 430904) and human IFNβ (Invitrogen; Cat. No. 414101) were performed using manufacturer's instructions. Absorbance was read on a Spark microplate reader (Tecan).

Luminex analysis

U937 macrophages were plated in 96-well plates at $5x10^4$ cells/well. Cells were stimulated for 24 hours with $1x10^8$ pfu/well of purified Pf4 phage or equivalent volume ΔPf4 preparation and 100 ng/ml LPS (Invivogen, Cat. No. tlrl-eblps). Cells were centrifuged at 300 x *g* for 5 minutes and supernatant was collected and used for cytokine profiling through Luminex (The Human Immune Monitoring Center, Stanford). Human 89-plex kits were purchased from eBiosciences/Affymetrix and used according to the manufacturer's recommendations with modifications as described below. Briefly: Beads were added to a 96 well plate and washed in a Biotek ELx405 washer. Samples were added to the plate containing the mixed antibody-linked beads and incubated at room temperature for 1 hour followed by overnight incubation at 4°C with shaking. Cold and room temperature incubation steps were performed on an orbital shaker at 500-600 rpm. Following the overnight incubation plates were washed in a Biotek ELx405 washer and then biotinylated detection antibody added for 75 minutes at room temperature with shaking. Plate was washed as

above and streptavidin-PE was added. After incubation for 30 minutes at room temperature wash was performed as above and reading buffer was added to the wells. Each sample was measured in duplicate. Plates were read using a Luminex 200 instrument with a lower bound of 50 beads per sample per cytokine. Custom assay Control beads by Radix Biosolutions were added to all wells.

Luminex data analysis
Technical replicate MFI values were averaged for each sample and transformed into $\log_2$ fold-change with respect to the control LPS sample. Data was plotted as a heatmap using the pheatmap package (v1.0.12) in R.

Isolation and preparation of neutrophils
Human neutrophil isolation protocol was adapted from *Swamydas* et al. (2015). Briefly, whole blood was obtained from the Stanford Blood Center, diluted 1:1 in 1x PBS (Corning, Cat. No. 21-040-CV), and layered over a double Histopaque-based density gradient (density 1.119 g/ml (Sigma-Aldrich, Cat. No. 11191-6X100ML) and density 1.077 g/ml (Sigma-Aldrich, Cat. No. 10771-6X100ML). The suspension was centrifuged at 900 *x g* for 30 minutes at 22ºC with no brake, and neutrophils from the interface of the Histopaque-1119 and Histopaque-1077 layers were collected and washed in RPMI + 10% FBS. Count and viability of neutrophils was determined by trypan blue exclusion.

Neutrophil migration assay
Primary human neutrophils isolated as described were stained at room temperature for 20 minutes with 5 µM Calcein AM (BioLegend, Cat. No. 425201) and applied to the top well of a fluorescence-blocking 24-well transwell plate with 3 µm pores (Corning, Cat. No. 351156). Bottom wells were filled with conditioned media from U937 macrophages, and chemotaxis was tracked by plate bottom fluorescence using a Spark microplate reader (Tecan) over the course of 30 minutes.

Murine pneumonia model
Experiments were performed as described in *Secor* et al. (2017). Briefly, an isolated colony of *P. aeruginosa* PAO1 or PAO1ΔPf4 was grown to mid-exponential phase (OD600, 0.5) in 4 ml LB broth at 37°C with aeration. The PAO1 culture was infected with 100 µl of Pf4 stock (10^{10} pfu/ml) to promote phage production. These cultures were grown overnight at 37°C with aeration. On the following day, bacteria were pelleted by centrifugation at 6,000 x *g* for 10 minutes, washed 3x in sterile PBS, and resuspended in PBS to a final concentration of $3x10^8$ CFU/ml. Mice (8-10wk C57BL/6, male; The Jackson Laboratory) were intranasally inoculated with $1.5x10^7$ CFU/ml in 50uL PBS of each strain or PBS control.

Mice were sacrificed at 24 hours post infection. The lungs were lavaged with 0.8 ml sterile PBS as described previously (*Secor* et al., 2017). BAL fluid samples were pelleted, and supernatant was frozen for use in ELISA and qPCR assays. BAL cells were resuspended in 1 ml ACK lysis buffer 30 s, followed by washing in PBS with 2% foetal calf serum and 1mM EDTA (fluorescence-activated cell sorting [FACS] buffer). The cells were resuspended in 1 ml FACS buffer and stained with Zombie Near-IR Live/Dead (Biolegend, Cat. No. 423105), CD11b-Pacific Blue (Biolegend, Cat. No. 101223), CD11c-BV605 (Biolegend, Cat. No. 117333, CD45.2-BV650 (Biolegend, Cat. No. 109835), Ly6G-BV785 (Biolegend, Cat. No. 127645), Siglec F-PE (Biolegend, Cat. No. 155505), and

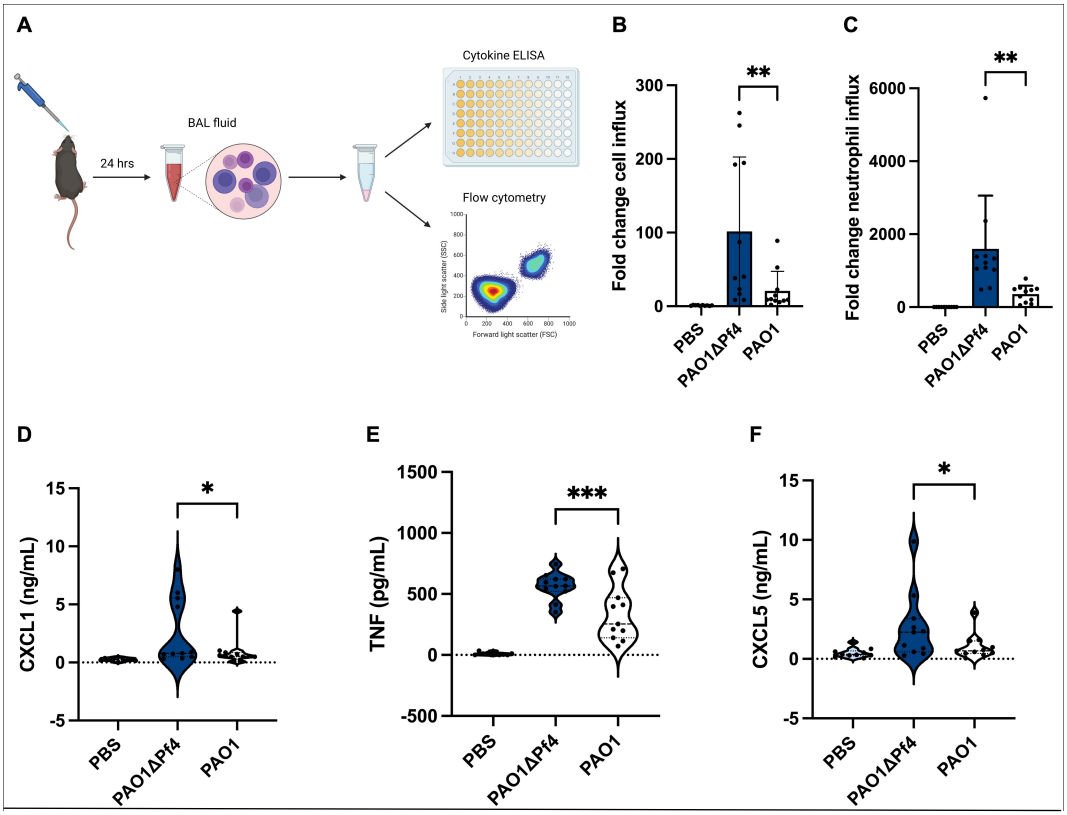


Figure 1: Neutrophil migration is reduced by Pf4 phage production in an acute *P. aeruginosa* pneumonia murine model.
(A) Mice were inoculated intranasally for 24 hours with either PBS, PAO1, or the isogenic *P. aeruginosa* strain PAO1ΔPf4 that lacks a functional copy of Pf4 phage. After 24 hours the animals were sacrificed and bronchoalveolar lavage (BAL) fluid was collected for cytokine measurements and cellular assessments via flow cytometry.
(B and C) BAL fluids samples were evaluated for (B) total cell content and (C) neutrophil quantities as determined by flow cytometry.
(D to E) BAL chemokine quantifications for (D) CXCL1, (E) TNF-α, and (F) CXCL5 as measured by ELISA.
Data are displayed as the mean ± SD from three individual experiments with n ≥ 9 mice per experiment. * = $p < 0.05$; ** = $p < 0.01$; and *** = $p < 0.001$. Significance was assessed by ANOVA followed by Holm-Šídák test for multiple comparisons.

Ly6C-AF647 (Biolegend, Cat. No. 128009) as per manufacturer's instructions. After staining, the cells were fixed with 4% paraformaldehyde and then run on a Cytek Northern Lights machine, followed by analysis with FlowJo™ software. Total live cells within the $CD45^+$ gate was reported, as well as live neutrophils within the $CD45^+$ $Ly6G^+$ gate. Absolute cell counts were adjusted by total BAL volume recovered.

Statistical analysis

Where n is not stated, graphs show a representative experiment of n ≥ 3 assays, with n ≥ 3 technical or biological replicates. All statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc. La Jolla, CA). Significance was assessed by ANOVA followed by Holm-Šídák test for multiple comparisons unless otherwise indicated. Depicted are means with standard

deviation (SD) of the replicates unless otherwise stated. Statistical significance was considered $p < 0.05$. Non-significance was indicated by the letters "n.s.". For experiments involving cell assays, each replicate was normalized against a specified control condition to control for varying inter-experiment intensities of cytokine production or reporter protein secretion.

RESULTS

Pf4-overproducing *P. aeruginosa* elicits diminished neutrophil migration

Building on our previous finding that treatment with *P. aeruginosa* strain PAO1 supplemented with Pf phage reduced pulmonary inflammation in response to bacterial infection (*Secor* et al., 2017), we examined *P. aeruginosa* infection in the absence of Pf4 phage. To this end, we treated mice with PAO1 or an isogenic strain lacking Pf4, PAO1ΔPf4. These strains have comparable growth curves (*Sweere* et al., 2019) such that differences between them cannot be attributed to altered growth. After 24 hours, bronchoalveolar lavage (BAL) fluid was collected and evaluated for cell influx and chemokine/cytokine content (Figure 1A). Mice infected with PAO1ΔPf4 showed significantly greater neutrophil influx and pro-inflammatory cytokine production than mice infected with Pf4-producing PAO1 (Figure 1B-C). PAO1-infected mice also exhibited significantly lower levels of CXCL1, TNF-α, and CXCL5 in BAL as compared to PAO1ΔPf4-infected mice (Figure 1D-F).

Together, these data demonstrate that Pf4 is associated with diminished neutrophil influx in this model in conjunction with reduced production of pro-inflammatory cytokines.

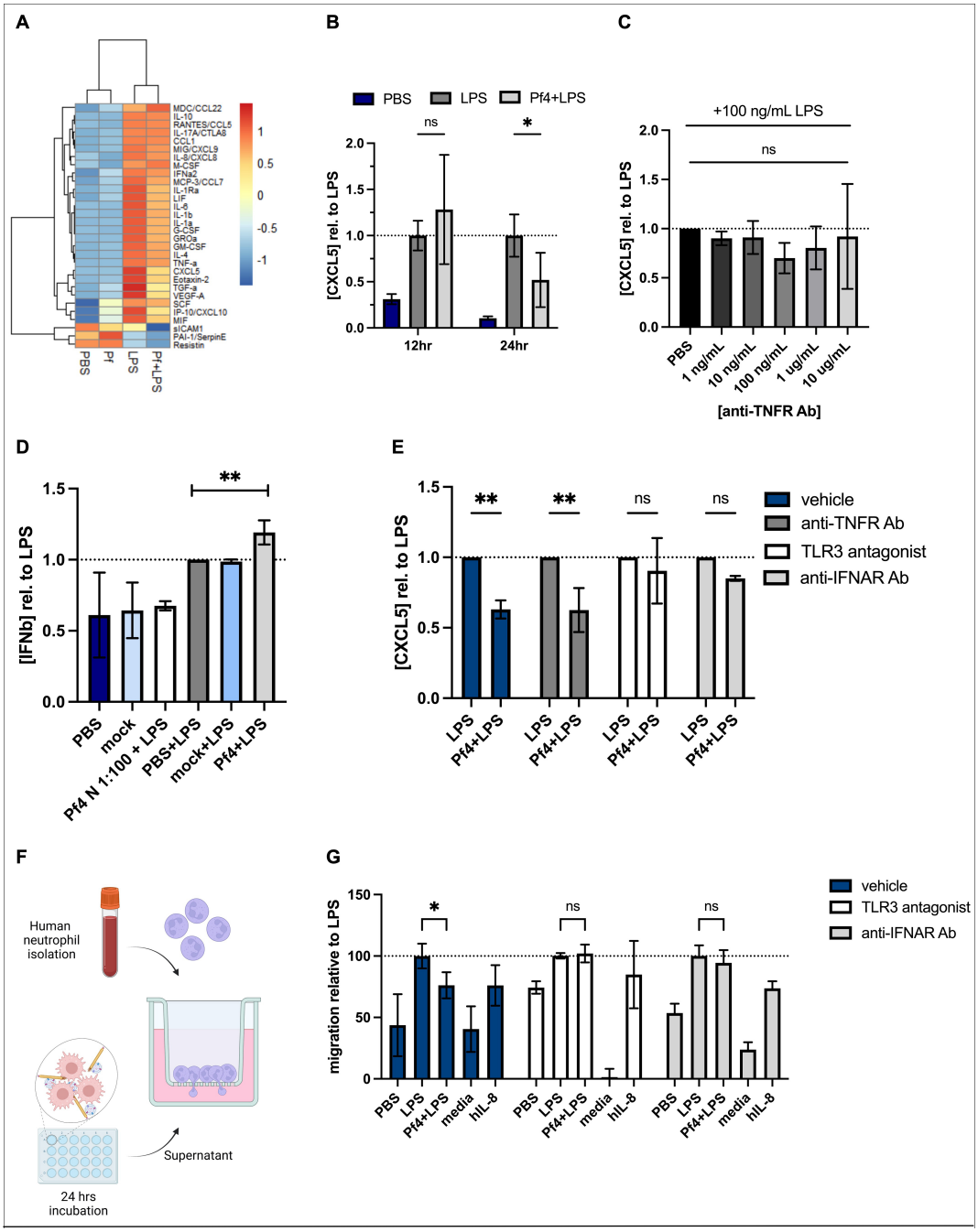
Pf4 downregulates a distinct set of neutrophil chemokines in a TLR3- and IFNAR-dependent manner

To obtain a more complete picture of Pf4-induced cytokine changes to the bacterial stimulation, we moved to an *in*

Figure 2: Pf4 impairs the production of a distinct set of chemokines in human macrophages which promote neutrophil migration in a TLR3- and IFNAR-dependent manner.
(A) Luminex analysis of U937 macrophage supernatants treated with 100 ng/ml LPS and/or 10^9 pfu/ml Pf4 for 24 hours.
(B and C) CXCL5 protein levels of U937 macrophages incubated with 100 ng/ml LPS, or 100 ng/ml LPS + 10^9 pfu/ml Pf4 at indicated time points. Anti-TNFR antibody was added in various concentrations to human U937 macrophages along with 100 ng/ml LPS and CXCL5 production was determined via ELISA.
(D) U937 human macrophages were supplemented with PBS, mock Pf4 preparation, or 10^9 pfu/ml Pf4 without or with 100 ng/ml LPS for 24 hours. IFNβ production was determined by ELISA.
(E) U937 cells treated with vehicle (PBS), 100 ng/ml anti-TNFR antibody, 27 μM of a TLR3/dsRNA complex inhibitor, or 100 ng/ml anti-IFNAR antibody along with LPS in absence or presence of Pf4 for 24 hours were assessed for CXCL5 production by ELISA.
(F) Schematic of human neutrophil migration assay. Human U937 macrophages were stimulated with LPS, Pf4 or TLR3 antagonists for 24 hours. The supernatant was isolated and used as a chemoattractant in a transwell setup. Human neutrophils isolated from freshly drawn blood and stained with Calcein AM, then applied to the top of a transwell plate with 3 μm pore size (STAR methods). Neutrophil migration towards conditioned media was assessed at 60 minutes.
(G) Neutrophil migration normalized to LPS control.
All data shown as the mean ± SD of at least 3 biological replicates with $n \geq 2$ technical replicates per condition and experiment. * = $p < 0.05$; ** = $p < 0.01$; and *** = $p < 0.001$. Significance was assessed by ANOVA followed by Holm-Šídák test for multiple comparisons.

vitro system with LPS as the source of inflammation. We examined supernatants from human U937 macrophages treated with LPS with or without Pf4 phage via multiplexed bead-based assay (Figure 2A). We used U937 cells for these experiments because of the central role of macrophages in airway inflammation (*Byrne* et al., 2015; *Hashimoto* et al., 1996).

We observed that Pf4 downregulated production of multiple factors made in



response to LPS including GROa/CXCL1, CXCL5, and TNF-α, as reported above, as well as other key pro-inflammatory proteins such as IL-6, IL-1a, IL-1b, and GM-CSF (Figure 2A). We chose to explore the functional impact of Pf4 on LPS-mediated production of CXCL5, a neutrophil chemoattractant, because dysregulation of neutrophil influx has been shown to strongly impact responses to *P. aeruginosa* and based on our findings in the acute *P. aeruginosa* lung model (*Koh* et al., 2009; *Lavoie* et al., 2011). Moreover, neutrophil influx is critical to control and clear *P. aeruginosa* infections (*Koh* et al., 2009; *Lavoie* et al., 2011).

We established that CXCL5 made in response to LPS is significantly down-regulated by Pf4 at the protein level by 24 hours in U937 macrophages, analogous to the effect of Pf4 phage on TNF-α production reported previously (*Sweere* et al., 2019) (Figure 2B). We then sought to determine whether CXCL5 levels reflect impaired TNF-α signaling in this system. To that end, we blocked the TNF-α receptor (TNFR) prior to treatment with Pf4. We found that CXCL5 protein expression is not significantly attenuated by diminished TNF-α signaling (Figure 2C).

We previously reported that TNF-α production by macrophages was inhibited by Pf phage in a TLR3 and type 1 interferon-dependent manner (*Sweere* et al., 2019). We asked whether CXCl5 inhibition was also mediated in this way. We indeed observed that Pf4 induces type I IFN production by macrophages (Figure 2D). We then assessed the dependency of the present system on TLR3 by treating U937 macrophages with a small-molecule compound that specifically prevents dsRNAs from binding TLR3 (*Cheng* et al., 2011), and found that CXCL5 down-regulation in response to Pf4 was abolished (Figure 2E). Similarly, cells treated with a blocking antibody against the type I IFN receptor, IFNAR1/2, showed a loss of phenotype (Figure 2E). Taken together, these data indicate that Pf4 downregulates multiple pro-inflammatory cytokines in human macrophages, including neutrophil chemoattractants, in a TLR3- and IFNAR-dependent manner. Moreover, the pathway that results in CXCL5 inhibition is parallel to but not dependent on the effects of Pf4 we previously reported on TNF-α (*Sweere* et al., 2019).

Pf4 impairs neutrophil recruitment by human macrophages

To test the functional consequences of reduced chemokine production, we adapted a neutrophil migration assay (*Frevert* et al., 1998) using freshly isolated human primary neutrophils from multiple donors and conditioned media from human U937 macrophages. Macrophages were exposed to purified Pf4 preparation along with LPS for 24 hours to induce inflammatory cytokine production, and conditioned media from these cells was used as a chemoattractant in a transwell setup (Figure 2F). Neutrophils were stained with the live-cell dye calcein AM and tracked as they migrated through the 3 μm pores of the transwell insert.

We found that conditioned media from cells treated with a combination of Pf4 and LPS induced less migration than cells treated with LPS alone (Figure 2G), indicating that the presence of purified Pf4 is sufficient to alter macrophage function in response to bacterial stimulation. Inhibition of either TLR3 or IFNAR in macrophages resulted in loss of Pf4-driven reduced neutrophil chemoattraction as compared to the LPS control (Figure 2G).

Together, these data indicate that Pf4 reduces the ability of LPS to stimulate macrophages to induce granulocyte migration.

DISCUSSION

We report that Pf4 phage downregulates multiple LPS-induced factors, including the potent neutrophil chemoattractant CXCL5. These suppressed macrophages are less effective at inducing neutrophil migration in a mouse model of acute *P. aeruginosa* lung infection, as well as *in vitro* using a human neutrophil migration assay. Neutrophil influx to sites of *P. aeruginosa* infection is critical to control and clear an infection (*Koh* et al., 2009; *Lavoie* et al., 2011). The findings we present in this work indicate that the presence of Pf4 phage could be a marker of negative outcome in patients infected with *P. aeruginosa*, through ineffective macrophage activation and subsequent impaired recruitment of neutrophils at early stages of infection. The intriguing question of whether Pf4-associated negative outcomes are related to the titres of Pf4 in the CF airways would be important to investigate further.

Although we chose to focus on the interaction between macrophages and neutrophils due to the observed downregulation of several neutrophil chemoattractants, several other cytokines altered by Pf4 stimulation may affect the course of an immune response to *P. aeruginosa*. In particular, the IL-1α/β and GM-CSF axis has been shown to be important for neutrophil longevity and thereby effective bacterial clearance (*Bober* et al., 1995; *Fossati* et al., 1998; *Laan* et al., 2003). In addition, GM-CSF has been shown to amplify bystander phagocyte cytokine production in *Legionella* infection (*Liu* et al., 2020).

The co-existence of filamentous phages with the bacterial hosts they infect suggests a potential symbiotic relationship. Indeed, prior work has demonstrated that Pf4 impacts *P. aeruginosa* pathogenesis during chronic pulmonary and wound infections (*Secor* et al., 2015; *Secor* et al., 2017; *Burgener* et al., 2019; *Sweere* et al., 2019). Unlike lytic phages which lyse the bacterial host cell, chronically infecting phages like Pf4 integrate their genetic material into the bacterial genome and co-exist with the bacteria in high titres. This co-existence assures that continued interaction between the phage and the bacterial cell will continue, manifesting in such phenomena as liquid crystal formation using bacterial polymers and phage-formed occlusive sheaths that protect bacterial cells from antibiotics (*Secor* et al., 2015; *Tarafder* et al., 2020). In this work, we present yet another instance of Pf4-*Pseduomonas* symbiosis, in which the phage prevents the clearance of the bacterium by reducing phagocyte immune responses.

ACKNOWLEDGMENTS

This work was funded by the grants from the NIH including R01 HL148184-01, R01 AI12492093, and R01 DC019965, as well as grants from the Cystic Fibrosis Foundation and the Emerson Collective. Funding support was also received from the National Science Foundation Graduate Research Fellowship (MCP, NH) and the Gabilan Stanford Graduate Fellowship (MCP, NH). Graphical schematics created with BioRender.com.

LITERATURE

Bille, E., Meyer, J., Jamet, A., Euphrasie, D., Barnier, J.-P., Brissac, T., Larsen, A., Pelissier, P., and Nassif, X.: A virulence-associated filamentous bacteriophage of Neisseria meningitidis increases host-cell colonisation. PLoS Pathog. 13, e1006495 (2017).

Bober, L.A., Grace, M.J., Pugliese-Sivo, C., Rojas-Triana, A., Waters, T., Sullivan, L.M., and Narula, S.K.: The effect of GM-CSF and G-CSF on human neutrophil function. Immunopharmacology 29, 111-119 (1995).

Burgener, E.B., Sweere, J.M., Bach, M.S., Secor, P.R., Haddock, N., Jennings, L.K., Marvig, R.L., Johansen, H.K., Rossi, E., Cao, X., Tian, L., Nedelec, L., Molin, S., Bollyky, P.L., and Milla, C.E.: Filamentous bacteriophages are associated with chronic Pseudomonas lung infections and antibiotic resistance in cystic fibrosis. Sci. Transl. Med. 11, eaau9748 (2019).

Byrne, A.J., Mathie, S.A., Gregory, L.G., and Lloyd, C.M.: Pulmonary macrophages: key players in the innate defence of the airways. Thorax 70, 1189-1196 (2015).

Cano, E.J., Caflisch, K.M., Bollyky, P.L., Van Belleghem, J.D., Patel, R., Fackler, J., Brownstein, M.J., Horne, B., Biswas, B., Henry, M., Malagon, F., Lewallen, D.G., and Suh, G.A.: Phage Therapy for Limb-threatening Prosthetic Knee Klebsiella pneumoniae Infection: Case Report and In Vitro Characterization of Anti-biofilm Activity. Clin. Infect. Dis. 73, e144-e151 (2021).

Cheng, K., Wang, X., and Yin, H.: Small-molecule inhibitors of the TLR3/dsRNA complex. J. Am. Chem. Soc. 133, 3764-3767 (2011).

Dąbrowska, K., Miernikiewicz, P., Piotrowicz, A., Hodyra, K., Owczarek, B., Lecion, D., Kaźmierczak, Z., Letarov, A., and Górski, A.: Immunogenicity studies of proteins forming the T4 phage head surface. J. Virol. 88, 12551-12557 (2014).

de Vries, C.R., Chen, Q., Demirdjian, S., Kaber, G., Khosravi, A., Liu, D., Van Belleghem, J.D., and Bollyky, P.L.: Phages in vaccine design and immunity; mechanisms and mysteries. Curr. Opin. Biotechnol. 68, 160-165 (2021).

Fiedoruk, K., Zakrzewska, M., Daniluk, T., Piktel, E., Chmielewska, S., and Bucki, R.: Two Lineages of Pseudomonas aeruginosa Filamentous Phages: Structural Uniformity over Integration Preferences. Genome Biol. Evol. 12, 1765-1781 (2020).

Fossati, G., Mazzucchelli, I., Gritti, D., Ricevuti, G., Edwards, S.W., Moulding, D.A., and Rossi, M.L.: In vitro effects of GM-CSF on mature peripheral blood neutrophils. Int. J. Mol. Med. 1, 943-994 (1998).

Frevert, C.W., Wong, V.A., Goodman, R.B., Goodwin, R., and Martin, T.R.: Rapid fluorescence-based measurement of neutrophil migration in vitro. J. Immunol. Methods 213, 41-52 (1998).

Hashimoto, S., Pittet, J.F., Hong, K., Folkesson, H., Bagby, G., Kobzik, L., Frevert, C., Watanabe, K., Tsurufuji, S., and Wiener-Kronish, J.: Depletion of alveolar macrophages decreases neutrophil chemotaxis to Pseudomonas airspace infections. Am. J. Physiol. 270, L819-28 (1996).

Hay, I.D. and Lithgow, T.: Filamentous phages: masters of a microbial sharing economy. EMBO Rep. 20, e47427 (2019).

Janmey, P.A., Slochower, D.R., Wang, Y.-H., Wen, Q., and Cēbers, A.: Polyelectrolyte properties of filamentous biopolymers and their consequences in biological fluids. Soft Matter 10, 1439-1449 (2014).

Knezevic, P., Voet, M., and Lavigne, R.: Prevalence of Pf1-like (pro)phage genetic elements among Pseudomonas aeruginosa isolates. Virology 483, 64-71 (2015).

Koh, A.Y., Priebe, G.P., Ray, C., Van Rooijen, N., and Pier, G.B.: Inescapable need for neutrophils as mediators of cellular innate immunity to acute Pseudomonas aeruginosa pneumonia. Infect. Immun. 77, 5300-5310 (2009).

Laan, M., Prause, O., Miyamoto, M., Sjöstrand, M., Hytönen, A.M., Kaneko, T., Lötvall, J., and Lindén, A.: A role of GM-CSF in the

accumulation of neutrophils in the airways caused by IL-17 and TNF-α. Eur. Respir. J. 21, 387-393 (2003).

Lavoie, E.G., Wangdi, T., and Kazmierczak, B.I.: Innate immune responses to Pseudomonas aeruginosa infection. Microbes Infect. 13, 1133-1145 (2011).

Liu, D., Van Belleghem, J.D., de Vries, C.R., Burgener, E., Chen, Q., Manasherob, R., Aronson, J.R., Amanatullah, D.F., Tamma, P.D., and Suh, G.A.: The Safety and Toxicity of Phage Therapy: A Review of Animal and Clinical Studies. Viruses 13, 1268 (2021).

Liu, X., Boyer, M.A., Holmgren, A.M., and Shin, S.: Legionella-Infected Macrophages Engage the Alveolar Epithelium to Metabolically Reprogram Myeloid Cells and Promote Antibacterial Inflammation. Cell Host Microbe 28, 683-698.e6 (2020).

Mai-Prochnow, A., Hui, J.G.K., Kjelleberg, S., Rakonjac, J., McDougald, D., and Rice, S.A.: 'Big things in small packages: the genetics of filamentous phage and effects on fitness of their host.' FEMS Microbiol. Rev. 39, 465-487 (2015).

Marvin, D.A., Symmons, M.F., and Straus, S.K.: Structure and assembly of filamentous bacteriophages. Prog. Biophys. Mol. Biol. 114, 80-122 (2014).

Pant, A., Das, B., and Bhadra, R.K.: CTX phage of Vibrio cholerae: Genomics and applications. Vaccine 38, Suppl. 1, A7-A12 (2020).

Popescu, M., Van Belleghem, J.D., Khosravi, A., and Bollyky, P.L.: Bacteriophages and the Immune System. Annu. Rev. Virol. 8, 415-35 (2021).

Pourtois, J.D., Kratochvil, M.J., Chen, Q., Haddock, N.L., Burgener, E.B., De Leo, G.A., and Bollyky, P.L.: Filamentous Bacteriophages and the Competitive Interaction between Pseudomonas aeruginosa Strains under Antibiotic Treatment: a Modeling Study. mSystems 6, e0019321 (2021).

Rice, S.A., Hao Tan, C., Jensen Mikkelsen, P., Kung, V., Woo, J., Tay, M., Hauser, A., McDougald, D., Webb, J.S., and Kjelleberg, S.: The biofilm life cycle and virulence of Pseudomonas aeruginosa are dependent on a filamentous prophage. ISME J. 3109, 271-282 (2009a).

Rice, S.A., Hao Tan, C., Mikkelsen, J., Kung, V., Woo, J., Tay, M., Hauser, A., Mcdougald, D., Webb, J.S., and Kjelleberg, S.: The biofilm life cycle and virulence of Pseudomonas aeruginosa are dependent on a filamentous prophage. ISME J. 3, 271-282 (2009b).

Roux, S., Krupovic, M., Daly, R.A., Borges, A.L., Nayfach, S., Schulz, F., Sharrar, A., Matheus Carnevali, P.B., Cheng, J.F., Ivanova, N.N., Bondy-Denomy, J., Wrighton, K.C., Woyke, T., Visel, A., Kyrpides, N.C., and Eloe-Fadrosh, E.A.: Cryptic inoviruses revealed as pervasive in bacteria and archaea across Earth's biomes. Nat. Microbiol. 4, 1895-1906 (2019).

Schmidt, A.K., Fitzpatrick, A.D., Schwartzkopf, C.M., Faith, D.R., Jennings, L.K., Coluccio, A., Hunt, D.J., Michaels, L.A., Hargil, A., Chen, Q., Bollyky, P.L., Dorward, D.W., Wachter, J., Rosa, P.A., Maxwell, K.L., and Secor, P.R.: A Filamentous Bacteriophage Protein Inhibits Type IV Pili To Prevent Superinfection of Pseudomonas aeruginosa. mBio. 13, e0244121 (2022).

Secor, P.R., Sweere, J.M., Michaels, L.A., Lazzareschi, D., Katznelson, E., Rajadas, J., Birnbaum, M.E., Arrigoni, A., Braun, K.R., Evanko, S.P., Stevens, D.A., Kaminsky, W., Singh, P.K., Parks, W.C., and Bollyky, P.L.: Filamentous Bacteriophage Promote Biofilm Assembly and Function. Cell Host Microbe 18, 549-559 (2015).

Secor, Patrick R, Michaels, L.A., Smigiel, K.S., Rohani, M.G., Jennings, L.K., Hisert, K.B., Arrigoni, A., Braun, K.R., Birkland, T.P., Lai, Y., Hallstrand, T.S., Bollyky, P.L., Singh, P.K., and Parks, W.C.: Filamentous Bacteriophage Produced by Pseudomonas aeruginosa Alters the Inflammatory Response and Promotes Noninvasive Infection In Vivo. Infect. Immun. 85, e00648-16 (2017).

Secor, P.R., Burgener, E.B., Kinnersley, M., Jennings, L.K., Roman-Cruz, V., Popescu, M., Van Belleghem, J.D., Haddock, N., Copeland, C., Michaels, L.A., de Vries,

C.R., Chen, Q., Pourtois, J., Wheeler, T.J., Milla, C.E., and Bollyky, P.L.: Pf Bacteriophage and Their Impact on *Pseudomonas* Virulence, Mammalian Immunity, and Chronic Infections. Front. Immunol. 11, 1-18 (2020).

Swamydas, M., Luo, Y., Dorf, M.E., and Lionakis, M.S.: Isolation of mouse neutrophils. Curr. Protoc. Immunol. 110, 3.20.1-3.20.15 (2015).

Sweere, J.M., Van Belleghem, J.D., Ishak, H., Bach, M.S., Popescu, M., Sunkari, V., Kaber, G., Manasherob, R., Suh, G.A., Cao, X., de Vries, C.R., Lam, D.N., Marshall, P.L., Birukova, M., Katznelson, E., Lazzareschi, D. V., Balaji, S., Keswani, S.G., Hawn, T.R., Secor, P.R., and Bollyky, P.L.: Bacteriophage trigger antiviral immunity and prevent clearance of bacterial infection. Science 363(6434), eaat9691 (2019).

Tarafder, A.K., von Kügelgen, A., Mellul, A.J., Schulze, U., Aarts, D.G.A.L., and Bharat, T.A.M.: Phage liquid crystalline droplets form occlusive sheaths that encapsulate and protect infectious rod-shaped bacteria. Proc. Natl. Acad. Sci. USA 117, 4724-4731 (2020).

TRIPARTITE INTERACTIONS BETWEEN BACTERIOPHAGES AND THEIR BACTERIAL AND MAMMALIAN HOSTS

JEREMY J. BARR

School of Biological Sciences, Monash University, Clayton Campus,
25 Rainforest Walk, Clayton, Victoria 3800, Australia

SUMMARY

Bacteriophages are obligate bacterial viruses capable of infecting and replicating only within their bacterial hosts. Yet this classical definition is limiting when we begin to consider bacteriophages within the broader context of their mammalian or eukaryotic hosts. Within this tripartite context bacteriophages may directly interact and influence their bacterial hosts, but they can further bind, enter, and stimulate the mammalian host directly. These interactions are largely unexplored and there exists an enormous potential for discovery of diver mechanisms, feedback loops, and symbioses within these tripartite contexts.

LINEAR RELATIONSHIPS

Picking up any undergraduate microbiology textbook you will find the definition of a 'bacteriophage' something akin to "*a virus capable of infecting and replicating only within bacterial cells*". This description applies when considering the diverse array of interactions that bacteriophages (or simply phages for short) can have with their bacterial hosts. These interactions span the diversity of symbioses including strictly parasitic through to mutualism. While this definition is technically correct, it is limiting when considering bacteriophages in a broader context of tripartite symbioses. In these tripartite systems bacteriophages may indeed directly interact with their bacterial hosts, but they also interact with their mammalian or eukaryotic host through a diverse range of mechanisms (Figure 1). These interactions can include direction binding to eukaryotic cells, in a fashion similar to their bacterial host, yet without the injection of their genetic material (*Lehti* et al., 2017). This was demonstrated by Lehti et al. showing that an *Escherichia coli* infecting bacteriophage that recognised a polysialic acid residue on its bacterial host could also target and bind the same residue on a eukaryotic neuroblastoma cell, triggering receptor mediated endocytosis and internalisation. Phages can also non-specifically adsorb or adhere to eukaryotic cell layers or their secretions (i.e. mucins) and be subsequently internalised through non-specific micropinocytosis events (*Barr* et al., 2013; *Bichet* et al., 2021a; *Nguyen* et al., 2017). Binding or directly adhering to cellular mucins may further elicit intracellular responses (*Barr*, 2017). This was demonstrated by Bloch et al. who characterised the interactions between phages and malignant tumour cells, showing that phages bound externally displayed mucins and inhibited the growth of these tumours (*Bloch*, 1940). Decades later, Dabrowska et al.

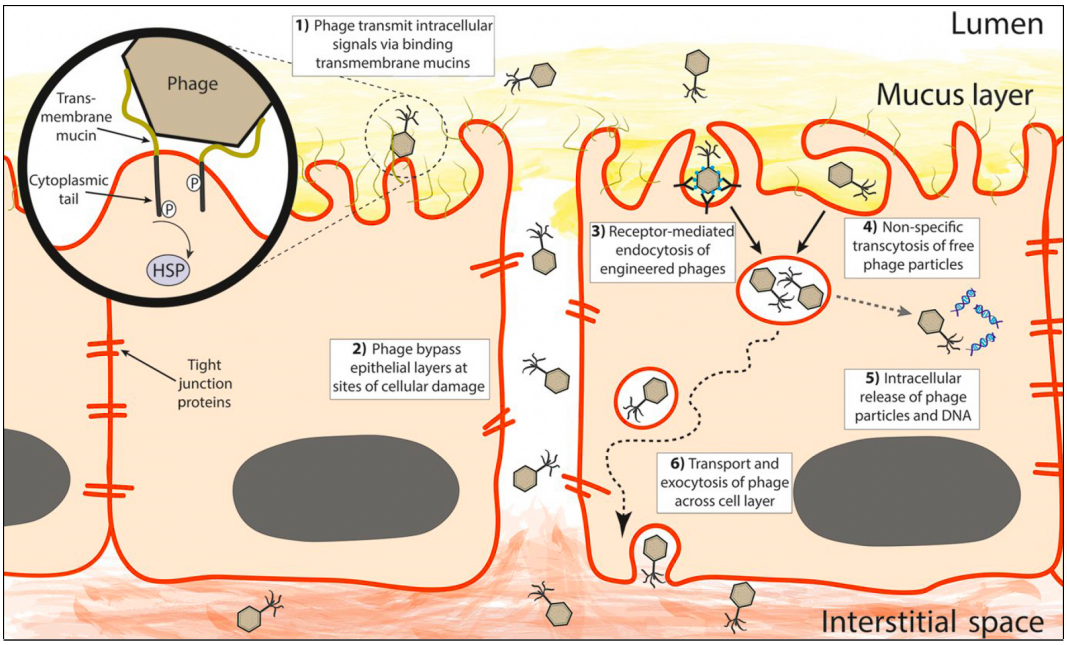


Figure 1: Bacteriophage interaction with the mammalian cell layer depicting the broad mechanism that phages can interact and stimulate the mammalian host directly. (Taken from Barr, 2017).

tested this observation, showing that T4 phages bound the membranes of cancer cells, attenuating tumour growth (*Dabrowska* et al., 2004a,b). Further, phages may indirectly stimulate or inhibit mammalian host cells and tissues through the production of auxiliary metabolic compounds encoded within their genomes and produced during either lytic or lysogenic infections of their bacterial hosts (*Sanchez* et al., 2015; *Thompson* et al., 2011). Indeed, the diversity of auxiliary metabolic genes that phages encode is highly diverse, with the production of these compounds having potentially varied effects. As such, it is important that we consider bacteriophages as not only 'viruses capably of infecting bacterial cells' but also as diverse biological agents capable of interacting with and influencing their broader mammalian hosts.

TRIPARTITE SYMBIOSES

Here I will discuss the concept of tripartite symbioses whereby bacteriophages can both directly and indirectly interact with their bacterial and mammalian hosts. This description is set in place of the previously described linear model, where bacteriophages were limited to only direct interactions with their bacterial hosts and indirect interactions with the mammalian host. Utilising this new tripartite model allows us to consider much broader affects those bacteriophages can have on larger symbiotic systems.

Taking the naturally-occurring phage populations residing within the human body we can consider the diversity of mechanisms by which they may

interact with both bacterial and mammalian hosts. In most cases, the mucosal surface is the first point of contact between the mammalian cell layer and phage populations that naturally reside within and upon it. Once there, bacteriophages can adhere to the mucosal surface (*Barr* et al., 2013; 2015). By adhering, bacteriophages can find and infect the resident bacterial community with greater ease. Once past the mucosa, phages can directly access the cell surface and interact with the underlying cellular epithelium. It has been demonstrated that phages are even capable of crossing this epithelial cell barrier in the gut and gain direct access to the bloodstream (*Górski* et al., 2006; *Nguyen* et al., 2017). This non-specific transcytosis mechanism has been proposed to facilitate 31 billion bacteriophage transcytosis events across the human gut epithelial barrier every day (*Nguyen* et al., 2017). Once within the circulatory system these 'intra-body phages' are able to gain access to all cells, organs and systems of the body (*Barr*, 2017). In fact it has been shown that some of these phages are even capable of crossing the blood-brain barrier – the most stringent biological barrier within the human body that even some small molecules and drugs fail to cross (*Geier* et al., 1973).

This tripartite model of bacteriophage-bacterial-mammalian interactions has led to a wave of research that has highlighted the diverse and surprising ways that bacteriophages can interact with and influence mammalian cells and those of other higher vertebrates. Bacteriophages have been demonstrated to bind cellular receptors on the apical surface of epithelial cell layers, leading to the activation of signal transduction pathways and other cellular functions (*Lehti* et al., 2017; *Singh* and *Hollingsworth*, 2006). These bacteriophage-cellular binding effects have been associated with increased mucus production, activation of anti-inflammatory responses, and even the aforementioned attenuation of tumour growth (*Dabrowska* et al., 2004; *Van Belleghem* et al., 2017). Further evidence suggests that phages are endocytosed by epithelial cells and trafficked throughout the endo-membrane system (*Lehti* et al., 2017; *Nguyen* et al., 2017). Once internalised, phages are encaged within membrane-bound vesicles. Once here, phage proteins and/or nucleic acids may be recognised by either cytostolic or endosomal membrane-bound receptors, triggering a broad host of cellular responses. But from where do these phages originate, and how can they build upon these tripartite models?

THE INTRA-BODY PHAGEOME

In a previous review article, I introduced and discussed the role of the 'intra-body phageome' (*Barr*, 2017). This phageome is proposed to originate from the highly diverse, expansive, and naturally occurring bacteriophage populations that are resident within the human gut. The gut microbiome plays an essential role in modulating our overall health and disease (*Scarpellini* et al., 2015). While the bacterial component of the gut microbiome has received considerable attention, comparatively little research and understanding has been provided to the gut viruses. Consisting overwhelmingly of bacteriophages, the gut virome is noted to contribute increasingly important roles in our overall health and well-being (*Clooney* et al., 2019; *Gregory* et al., 2020; *Liang* et al., 2020; *Sutton* and *Hill*, 2019). Gut bacteriophages can directly predate upon and regulate gut bacterial populations. Further, gut

phages can indirectly influence gut bacterial populations through the opening of niche space, release of diverse metabolites, and in-direct modulation of inter-bacterial species competition (*Hsu* et al., 2019). There have been a number of studies investigating the role of gut bacteriophages in states of dysbiosis. As such it has been found that specific bacteriophage populations have been correlated with a range of inflammatory bowel diseases, such as Crohn's and Ulcerative Colitis (*Norman* et al., 2015; *Clooney* et al., 2019). Conversely, gut bacteriophages have also been linked with a number of beneficial implications, including a reported increase in cognitive function through the modulation of bacterial populations and the secretion of key neuro-transmitters in the gut, which were found to affect short-term memory in flies and mice, while correlations were seen within human cohorts (*Maynernis-Percaxhs* et al., 2022). As such, there is a growing body of evidence suggestion the potential health and disease benefits of these gut bacteriophages.

Importantly, these resident gut bacteriophage populations with their potential health and immune-modulatory affects are also the source for the 'intra-body phageome'. Here, gut bacteriophage populations interact with the epithelial cell layers of the large intestine and subsequently are internalised by non-specific macropinocytosis mechanisms (*Bichet* et al., 2021b; *Nguyen* et al., 2017). This was proposed to facilitate over 31 billion phage uptake and transcytosis events within an adult human every single day (*Nguyen* et al., 2017). Thus, there exists a large potential for resident gut bacteriophages to be continually internalised and trafficked into the 'classically-sterile' regions of the body. This allows for a low-level, constituent resident collection of naturally occurring gut bacteriophages that are interacting with the broader mammalian host cells, organs and systems. This broader tripartite system has incredible potential to modulate the mammalian host in diverse and largely as yet undiscovered ways.

PHAGE UPTAKE, DELIVERY AND ACTIVATION OF THE MAMMALIAN SYSTEM

The broader question when considering tripartite symbioses is 'why do bacteriophages interact with mammalian cells' and 'what potential response could they be mediating?' (Or, if you're proclivity is mammalian-centric, 'why do mammalian cells internalise bacteriophages and for what purpose?'). The answer to this question could be surprising, diverse, and unexpected. In fact, these interactions and their derivative effects may be so unexpected that it would be foolish to predict these entirely. Instead, we should look to the diversity of interactions that the gut bacteria mediate and the process towards their discoveries. Here I will explore two potential mechanisms that bacteriophages can and may directly influence the mammalian host.

The first is through the triggering of pattern recognition receptors (PRRs). As bacteriophages contain either RNA or DNA genomes as part of their life cycle, the nucleic acid sensing of the mammalian cell could be triggered. Once bacteriophage particles are internalised by mammalian cells there exist two main mechanisms through which

their genomes could trigger PRRs (*Tan* et al., 2018). The first is through TLR9 receptors positioned within the endosomal structures. It has been shown that bacteriophage particles are endocytosed and trafficked through the endomembrane system (*Bichet*, et al., 2021a; *Nguyen* et al., 2017). If bacteriophage capsids are damaged or tail fiber structures triggered, then their genomic material could be exposed within the endosomes and lead to the activation of TLR9, whose downstream activation can stimulate Type I IFN response (*Sweere* et al., 2019; *Van Belleghem* et al., 2019). Alternatively, if bacteriophage particles or their genomes escape these endomembrane vesicles, they may gain access to the mammalian cytosol. There the major innate immune sensor for nucleic acids is the cGAS/STING (cyclic GMP-AMO synthase/stimulator of interferon genes) pathway (*Tan* et al., 2018). When dsDNA fragments are detected by cGAS, a molecule of cGAMP will be produced and sent to the STING complex to activate the production of cytokines like INF (*Blasius* and *Beutler*, 2010). These two pathways lead to signalling cascades that culminate in the production of inflammatory cytokines, leading to the induction of an antimicrobial state, activation of adaptive immunity, and eventual clearance of the triggering pathogen.

The second potential mechanism that bacteriophages could influence the mammalian cell is through direct protein-to-protein interactions. This could be initiated through bacteriophage capsid interactions with G-protein coupled receptors at the cell membrane (*Bosch* et al., 2009). Once activated these could lead to protein phosphorylation cascade within the cell that may activate a diverse array of mechanistic responses and modulation of cellular function (*Carraway* et al., 2003; *Lillehoj* et al., 2004). Mechanistically these two responses are diverse in both their activation and downstream signal cascades. Further experimental validation of if and how bacteriophages may interact with mammalian cells and the responses they induce are needed.

HYPOTHETICAL GENE POTENTIAL

A final hypothetical alternative for bacteriophage uptake and internalisation by mammalian cells is the potential for gene delivery and transduction. Once internalised, there exists the possibility for bacteriophages to deliver their genetic material into the mammalian cell allowing for the transcription and translation of virally encoded DNA by the eukaryotic cellular machinery – a process more broadly known as 'transduction' (*Merril*, 1974; *Tao* et al., 2013).

Bacteriophages have indeed been used as viral gene delivery vectors and nanocarriers, primarily due to their ease of use, capacity for nucleic acid packaging and their relative safety in humans (*Karimi* et al., 2016). To accomplish this, a process known as 'phage-display' is used for the targeted delivery of phage-carried nucleic acids and proteins to specific mammalian cells (*Ivanenkov* and *Menon*, 2000; *Pranjol* and *Hajitou*, 2015). In this process bacteriophages are engineered to display ligands on their capsids that are complementary to mammalian cell surface integrins. Phage-displayed ligands then bind to these integrins and trigger receptor-mediated endocytosis of the bacteriophage particle on contact with the mammalian cell. These bacteriophage capsids can be recombinantly packaged with DNA, RNA, or proteins for the targeted delivery of genes and

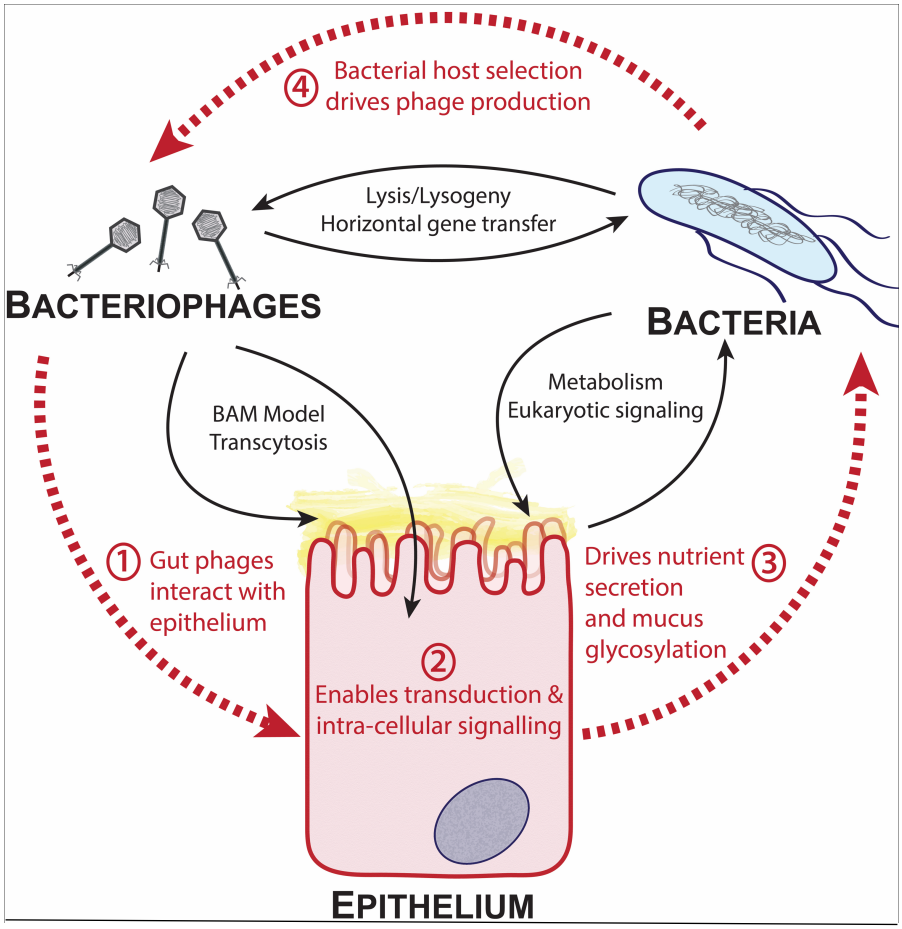


Figure 2: Model of tripartite symbioses in the human gut and the potential for the delivery and transduction of bacteriophage-encoded nucleic acid material to the mammalian cell. (Taken from Barr, 2019).

enzymes into the mammalian cells of interest. Using these approaches, engineered bacteriophage particles have successfully been used to transduce mammalian cells to correct metabolic deficiencies (*Geier* et al., 1973; *Merril* et al., 1971), elicit antibody response (*Tao* et al., 2013), and to deliver reporter genes or enzymes (*Poul* and *Marks*, 1999). These studies demonstrate the capacity for bacteriophage-encoded genetic material to be delivered to and transduce mammalian cells.

This leads to a major hypothesis as to whether the uptake and internalisation of naturally occurring bacteriophage populations within the human gut are capable of transduction (Figure 2). As theses gut bacteriophages are known to be internalised and transcytosed at consistent levels (*Nguyen* et al., 2017) and studies have shown engineered bacteriophage particles can deliver and transduce cells (*Geier* and *Merril*, 1972) this mechanistic route for natural bacteriophage populations to transduce the mammalian host remains and open possibility.

When considering the enormous diversity of bacteriophage populations within the human gut and the large

hypothetical genes they encode – more commonly referred to as 'viral dark matter' – this is an intriguing hypothesis to explore further. A clear understanding of the cellular and molecular interactions between these bacteriophage particles and mammalian cells will be required to elucidate any novel symbioses.

LITERATURE

Barr, J.J., Auro, R., Furlan, M., Whiteson, K. L. L., Erb, M. L. L., Pogliano, J., Stotland, A., Wolkowicz, R., Cutting, A. S. S., Doran, K. S. S., Salamon, P., Youle, M., and Rohwer, F.: Bacteriophage adhering to mucus provide a non-host-derived immunity. Proc. Natl. Acad. Sci. USA 110, 10771-10776 (2013).

Barr, J.J., Auro, R., Sam-Soon, N., Kassegne, S., Peters, G., Bonilla, N., Hatay, M., Mourtada, S., Bailey, B., Youle, M., Felts, B., Baljon, A., Nulton, J., Salamon, P., and Rohwer, F.: Subdiffusive motion of bacteriophage in mucosal surfaces increases the frequency of bacterial encounters. Proc. Natl. Acad. Sci. USA 112, 13675-13680 (2015).

Barr, J.J.: A bacteriophages journey through the human body. Immunol. Rev. 279, 106-122 (2017).

Barr, J.J.: Missing a Phage: Unraveling Tripartite Symbioses within the Human Gut. mSystems 7, e00105-19 (2019).

Bichet, M.C., Chin, W.H., Richards, W., Lin, Y.-W., Avellaneda-Franco, L., Hernandez, C.A., Oddo, A., Chernyavskiy, O., Hilsenstein, V., Neild, A., Li, J., Voelcker, N.H., Patwa, R., and Barr, J.J.: Bacteriophage uptake by mammalian cell layers represents a potential sink that may impact phage therapy. iScience 24, 102287 (2021a).

Bichet, M.C., Patwa, R., and Barr, J.J.: Protocols for studying bacteriophage interactions with *in vitro* epithelial cell layers. STAR Protoc. 2, 100697 (2021b).

Blasius, A.L. and Beutler, B.: Intracellular Toll-like Receptors. Immunity 32, 305-315 (2010).

Bloch, H.: Experimental investigation on the relationship between bacteriophages and maligant tumors. Arch. Virol. 1, 481-496 (1940).

Bosch, T.C.G., Augustin, R., Anton-Erxleben, F., Fraune, S., Hemmrich, G., Zill, H., Rosenstiel, P., Jacobs, G., Schreiber, S., Leippe, M., Stanisak, M., Grötzinger, J., Jung, S., Podschun, R., Bartels, J., Harder, J., and Schröder, J.-M.: Uncovering the evolutionary history of innate immunity: the simple metazoan Hydra uses epithelial cells for host defence. Dev. Comp. Immunol. 33, 559-569 (2009).

Carraway, K.L., Ramsauer, V.P., Haq, B., and Carothers Carraway, C.A.: Cell signaling through membrane mucins. Bioessays 25, 66-71 (2003).

Clooney, A.G., Sutton, T.D.S., Shkoporov, A.N., Holohan, R.K., Daly, K.M., O'Regan, O., Ryan, F.J., Draper, L.A., Plevy, S.E., Ross, R.P., and Hill, C.: Whole-Virome Analysis Sheds Light on Viral Dark Matter in Inflammatory Bowel Disease. Cell Host Microbe 26, 764-778 (2019).

Dabrowska, K, Opolski, A., Wietrzyk, J., Switala-Jelen, K., Boratynski, J., Nasulewicz, A., Lipinska, L., Chybicka, A., Kujawa, M., Zabel, M., Dolinska-Krajewska, B., Piasecki, E., Weber-Dabrowska, B., Rybka, J., Salwa, J., Wojdat, E., Nowaczyk, M., and Gorski, A.: Antitumor activity of bacteriophages in murine experimental cancer models caused possibly by inhibition of beta3 integrin signaling pathway. Acta Virol. 48, 241-248 (2004a).

Dabrowska, K., Opolski, A., Wietrzyk, J., Switala-Jelen, K., Godlewska, J., Boratynski, J., Syper, D., Weber-Dabrowska, B., and Gorski, A.: Anticancer activity of bacteriophage T4 and its mutant HAP1 in

mouse experimental tumour models. Anticancer Res. 24, 3991-3995 (2004b).
Geier, M.R. and Merril, C.R.: Lambda phage transcription in human fibroblasts. Virology 47, 638-643 (1972).
Geier, MR., Trigg, M.E., and Merril, C.R.: Fate of bacteriophage lambda in non-immune germ-free mice. Nature 246, 221-223 (1973).
Górski, A., Wazna, E., Dabrowska, B.-W., Dabrowska, K., Switała-Jeleń, K., and Miedzybrodzki, R.: Bacteriophage translocation. FEMS Immunol. Med. Microbiol. 46, 313-319 (2006).
Gregory, A.C., Zablocki, O., Zayed, A.A., Howell, A., Bolduc, B., and Sullivan, M.B.: The Gut Virome Database Reveals Age-Dependent Patterns of Virome Diversity in the Human Gut. Cell Host Microbe 28, 724-740 (2020).
Hsu, B.B., Gibson, T.E., Yeliseyev, V., Liu, Q., Lyon, L., Bry, L., Silver, P.A., and Gerber, G.K.: Dynamic Modulation of the Gut Microbiota and Metabolome by Bacteriophages in a Mouse Model. Cell Host Microbe *25*, 803-814 (2019).
Ivanenkov, V.V. and Menon, A.G.: Peptide-mediated transcytosis of phage display vectors in MDCK Cells. Biochem. Biophys. Res. Commun. 276, 251-257 (2000).
Karimi, M., Mirshekari, H., Moosavi Basri, S.M., Bahrami, S., Moghoofei, M., and Hamblin, M.R.: Bacteriophages and phage-inspired nanocarriers for targeted delivery of therapeutic cargos. Adv. Drug Deliv. Rev. 106, 45-62 (2016).
Lehti, T.A., Pajunen, M.I., Skog, M.S., and Finne, J.: Internalization of a polysialic acid-binding Escherichia coli bacteriophage into eukaryotic neuroblastoma cells. Nature Commun. 8, 1915 (2017).
Liang, G., Zhao, C., Zhang, H., Mattei, L., Sherrill-Mix, S., Bittinger, K., Kessler, L.R., Wu, G.D., Baldassano, R.N., DeRusso, P., Ford, E., Elovitz, M.A., Kelly, M.S., Patel, M.Z., Mazhani, T., Gerber, J.S., Kelly, A., Zemel, B.S., and Bushman, F.D.: The stepwise assembly of the neonatal virome is modulated by breastfeeding. Nature 581, 470-474 (2020).
Lillehoj, E.P., Kim, H., Chun, E.Y., and Kim, K.C.: Pseudomonas aeruginosa stimulates phosphorylation of the airway epithelial membrane glycoprotein Muc1 and activates MAP kinase. Am. J. Physiol. Lung Cell. Mol. Physiol. 287, L809-L815 (2004).
Maynernis-Percaxhs, J., Castells-Nobau, A., Arnoriaga-Rodriguez, M., Maldonado, R., and Fernandez-Real, J.-M.: Caudovirales bacteriophages are associated with improved executive function and memory in flies, mice, and humans. Cell Host Microbe 30, 340-356 (2022).
Merril, C.R., Geier, M.R., and Petricciani, J.C.: Bacterial virus gene expression in human cells. Nature 233, 398-400 (1971).
Merril, C.R.: Bacteriophage interactions with higher organisms. Trans. NY Acad. Sci. 36, 265-272 (1974).
Nguyen, S., Baker, K., Padman, B.S., Patwa, R., Dunstan, R.A., Weston, T.A., Schlosser, K., Bailey, B., Lithgow, T., Lazarou, M., Luque, A., Rohwer, F., Blumberg, R.S., and Barr, J.J.: Bacteriophage Transcytosis Provides a Mechanism To Cross Epithelial Cell Layers. MBio, 8, e01874-17 (2017).
Norman, J.M., Handley, S.A., Baldridge, M.T., Droit, L., Liu, C.Y., Keller, B.C., Kambal, A., Monaco, C.L., Zhao, G., Fleshner, P., Stappenbeck, T.S., McGovern, D.P.B., Keshavarzian, A., Mutlu, E.A., Sauk, J., Gevers, D., Xavier, R.J., Wang, D., Parkes, M., and Virgin, H.W.: Disease-Specific Alterations in the Enteric Virome in Inflammatory Bowel Disease. Cell 160, 447-460 (2015).
Poul, M. a, & Marks, J. D.: Targeted gene delivery to mammalian cells by filamentous bacteriophage. J. Mol. Biol. 288, 203-211 (1999).
Pranjol, M.Z.I. and Hajitou, A.: Bacteriophage-derived vectors for targeted cancer gene therapy. Viruses 7, 268-284 (2015).
Sanchez, S.E., Cuevas, D.A., Rostron, J.E., Liang, T.Y., Pivaroff, C.G., Haynes, M.R., Nulton, J., Felts, B., Bailey, B.A., Salamon, P., Edwards, R.A., Burgin, A.B., Segall, A.M., and Rohwer, F.: Phage Phenomics:

Physiological Approaches to Characterize Novel Viral Proteins. J. Vis. Exp. 100: e52854 (2015).

Scarpellini, E., Ianiro, G., Attili, F., Bassanelli, C., De Santis, A., and Gasbarrini, A.: The human gut microbiota and virome: Potential therapeutic implications. Dig. Liver Dis. 47, 1007-1012 (2015).

Singh, P.K. and Hollingsworth, M.A.: Cell surface-associated mucins in signal transduction. Trends Cell. Biol. 16, 467-476 (2006).

Sutton, T.D.S. and Hill, C.: Gut Bacteriophage: Current Understanding and Challenges. Front. Endocrinol. 10, 784 (2019).

Sweere, J.M., Van Belleghem, J.D., Ishak, H., Bach, M.S., Popescu, M., Sunkari, V., Kaber, G., Manasherob, R., Suh, G.A., Cao, X., de Vries, C.R., Lam, D.N., Marshall, P.L., Birukova, M., Katznelson, E., Lazzareschi, D.V., Balaji, S., Keswani, S.G., Hawn, T., Secor, P.R., and Bollyky, P.L.: Bacteriophage trigger antiviral immunity and prevent clearance of bacterial infection. Science 363, eaat9691 (2019).

Tan, X., Sun, L., Chen, J., and Chen, Z.J.: Detection of Microbial Infections Through Innate Immune Sensing of Nucleic Acids. Annu. Rev. Microbiol. 72, 447-478 (2018).

Tao, P., Mahalingam, M., Marasa, B.S., Zhang, Z., Chopra, A.K., and Rao, V.B.: In vitro and in vivo delivery of genes and proteins using the bacteriophage T4 DNA packaging machine. Proc. Natl Acad. Sci. USA, 110, 5846-5851 (2013).

Thompson, L.R., Zeng, Q., Kelly, L., Huang, K.H., Singer, A.U., Stubbe, J., and Chisholm, S.W.: Phage auxiliary metabolic genes and the redirection of cyanobacterial host carbon metabolism. Proc. Natl Acad. Sci. USA, 108, E757-E764 (2011).

Van Belleghem, J.D., Clement, F., Merabishvili, M., Lavigne, R., and Vaneechoutte, M.: Pro- and anti-inflammatory responses of peripheral blood mononuclear cells induced by Staphylococcus aureus and Pseudomonas aeruginosa phages. Sci. Rep. 7, 8004 (2017).

Van Belleghem, J.D., Dąbrowska, K., Vaneechoutte, M., Barr, J. J., and Bollyky, P. L.: Interactions between bacteriophage, bacteria, and the mammalian immune system. Viruses 11: 10 (2019).

GUT VIROME CHANGES WITH NUTRITION AND METABOLIC DISEASE INFLUENCING COGNITIVE FUNCTION

ANNA MOTGER ALBERTÍ and JOSÉ MANUEL FERNÁNDEZ-REAL

Department of Diabetes, Endocrinology and Nutrition, Dr. Josep Trueta University Hospital, Avinguda de França, s/n, 17007 Girona, Spain; Department of Medical Sciences, Faculty of Medicine, Girona University, Edifici Centre, c/ Emili Grahit 77, Campus Centre, 17003 Girona, Spain; and Nutrition, Eumetabolism and Health Group, Girona Biomedical Research Institute (IdibGi), Carrer del Dr. Castany, s/n, 17190 Salt, Girona, Spain

SUMMARY

The study of the gut virome and its relationship with illness has emerged in recent years. Some studies have hinted at bacterial microbiome dysbiosis as having a substantial impact on the pathophysiology and development of metabolic diseases such obesity and type 2 diabetes. The gut microorganism ecology interacts with metabolic impairment and could play a role in the systemic traits of those prevalent metabolic diseases. Importantly, the richness and diversity of the gut virome is decreased in adult subjects with obesity. Furthermore, recent research has revealed a link between gut bacteria and cognition. *Caudovirales*, in particular, were associated with enhanced executive function and immediate memory. *Microviridae,* on the other hand, may be detrimental to cognitive function. Siphoviridae and Microviridae counts were associated with specific bacterial microbiome profile in four independent cohorts. Gut bacterial functions and plasma and faecal metabolites run in parallel to bacteriophage counts, integrated in a network that influenced cognition. A kind of dose-response effects of bacteriophages was observed in the human gut microbiome transplanted to mice: the genes that most changed in recipient mice were precisely those involved in memory in a concordant manner with mice cognition. These findings could open up new horizons in the pathophysiology of mental health and neurological diseases arising from the gut.

INTRODUCTION

Cognitive decline is becoming increasingly common as people live longer, and is one of the world's leading public health problems. The prevalence of dementia has risen sharply and is expected to increase by more than 78 million and 139 million by 2050. Obesity, on the other hand, is a metabolic disease of concern due to its exponential upward trend, and has become a serious public health issue in recent decades, with hundreds of millions of subjects showing overweight and obesity (WHO, 2019).

The ineffectiveness of existing approaches and therapies are compelling reasons to discover new ways to understand and treat them. One of the mechanisms that has recently gained strength for addressing and comprehending these disorders is the Gut-Brain Axis (GBA) approach, a bidirectional

Table 1: Cognitive function is divided into six cognitive domains by the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), each of which has subdomains

COGNITIVE DOMAINS	SUBDOMAINS	BRAIN AREA
Executive function	Planning, decision making, working memory, responding to feedback, inhibition, and flexibility.	Frontal lobe
Complex attention	Sustained, divided, selective attention and processing speed.	
Social recognition	Recognition of emotions, theory of mind, and Insight.	
Learning and memory	Free and cued recall, recognition memory, semantic and autobiographic, long-term memory, and implicit memory.	Temporal lobe
Language	Objects naming, word-finding, fluency, grammar and syntax and receptive language.	
Perceptual-motor function	Visuoconstructional reasoning and perceptual-motor coordination.	Parietal lobe
	Visual perception	Occipital lobe

communication system that connects the brain (emotional and cognitive processes) with peripheral intestinal functions.

Recent research breakthroughs have discovered the microbiome that influences these relationships. The microbiota influences gut-brain communication via endocrine, (*Fava* et al., 2019), immunological (*Hooper* et al., 2012) and neuroactive pathways (*Socała* et al., 2021). Microbial neurotransmitters (e.g. GABA, catecholamines) and metabolites such as Short Chain Fatty-Acids (SCFAs) (*Topping* et al., 2001; *Koh* et al., 2016), bile acids (2BAs) (*Jones* et al., 2008), and tryptophan (*O' Mahony* et al., 2015) are the most well-known examples of microbial-derived intermediates that communicate from the gut microbiome to the central nervous system (CNS). Although some of these intermediates directly interact with enteroendocrine cells, enterochromaffin cells, and the mucosal immune system to spread bottom-up signalling, others can pass the intestinal barrier and enter systemic circulation, and may even breach the blood-brain barrier (BBB). Microbial signals may also be sent through neurological pathways involving vagal and/or spinal afferents (*Latorre* et al., 2016).

In fact, this new gut-brain approach is rethinking the study of various diseases affecting the central nervous system, from neurodegenerative diseases such as Alzheimer's or Parkinson's, neurodevelopmental disorders such as Attention-Deficit Hyperactivity Disorder (ADHD), Autism spectrum disorder, or even psychiatric diseases such as depression (*Chen* et al., 2021). Previously, these diseases were approached from the perspective of brain mechanisms. However, in recent years, the focus of these diseases has shifted towards the gut and its microbiota, as well as its impact on the brain. Furthermore, not only can the aforementioned disorders influence cognition, but metabolic diseases like obesity also affect

cognitive functioning, especially executive skills and memory (*Arnoriaga-Rodríguez* et al., 2020, 2021). Moreover, cognitive dysfunction has been described as both a cause and a consequence of obesity.

In order to better understand the different cognitive functions, this chapter will provide a brief introduction and description of the cognitive domains according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-V). Then, the link between the gut and metabolic diseases will be explored. Lastly, current human results relating to cognition and the gut virome will be discussed.

BRAIN STRUCTURE AND COGNITIVE FUNCTIONS

Cognitive functions are processes in the CNS that take place in different areas of the brain, which in turn, are interconnected with each other. CNS integrates data from the entire body and coordinates activities throughout the whole organism.

The Diagnostic and Statistical Manual of Mental Disorders divides cognitive function into six cognitive domains, each of which has subdomains (*American Psychiatric Association*, 2013), which have been acknowledged by the neuropsychological and psychiatric societies.

The brain is divided into four cortical lobes: frontal lobe, temporal lobe, parietal lobe and occipital lobe. These are not only connected to each other, but also to subcortical structures (caudate nucleus, globus pallidus, amygdala, etc.). Despite this interconnectivity between areas, studies of brain damage have revealed which region of the brain is primarily responsible for each cognitive function, as well as emotion and behaviour processes.

The frontal lobe is involved in attention and executive function (working memory, inhibition, self-regulation, organization and planning, and phonemic verbal fluency). Executive function is a combination of cognitive abilities that enable us to manage our behaviour, set goals, and analyse information in order to become as adaptable as possible in our environment. This specific area is involved in psychiatric and movement disorders. The temporal lobe is implicated in language (processing and understanding verbal information and speaking among others) and memory function (storing and retrieving information). The parietal lobe is involved in processes and integrates sensory information, orientation, and visuospatial skills. Finally, the occipital lobe is involved in visuoperception, memory formation, and face recognition (Table 1). A neuropsychologist evaluates cognitive function using particular neurocognitive tests that produce a raw score that is then translated into normative-standard scores according to the test manual.

In the same way that establishing cognitive impairment in patients with neurological diseases is critical, assessing cognitive decline in patients with obesity and/or type 2 diabetes may explain disease maintenance. Therefore, understanding how cognitive impairment has an effect on these metabolic diseases could make it possible to find physical or dietary treatments (*Arnoriaga-Rodríguez* and *Fernández-Real*, 2019).

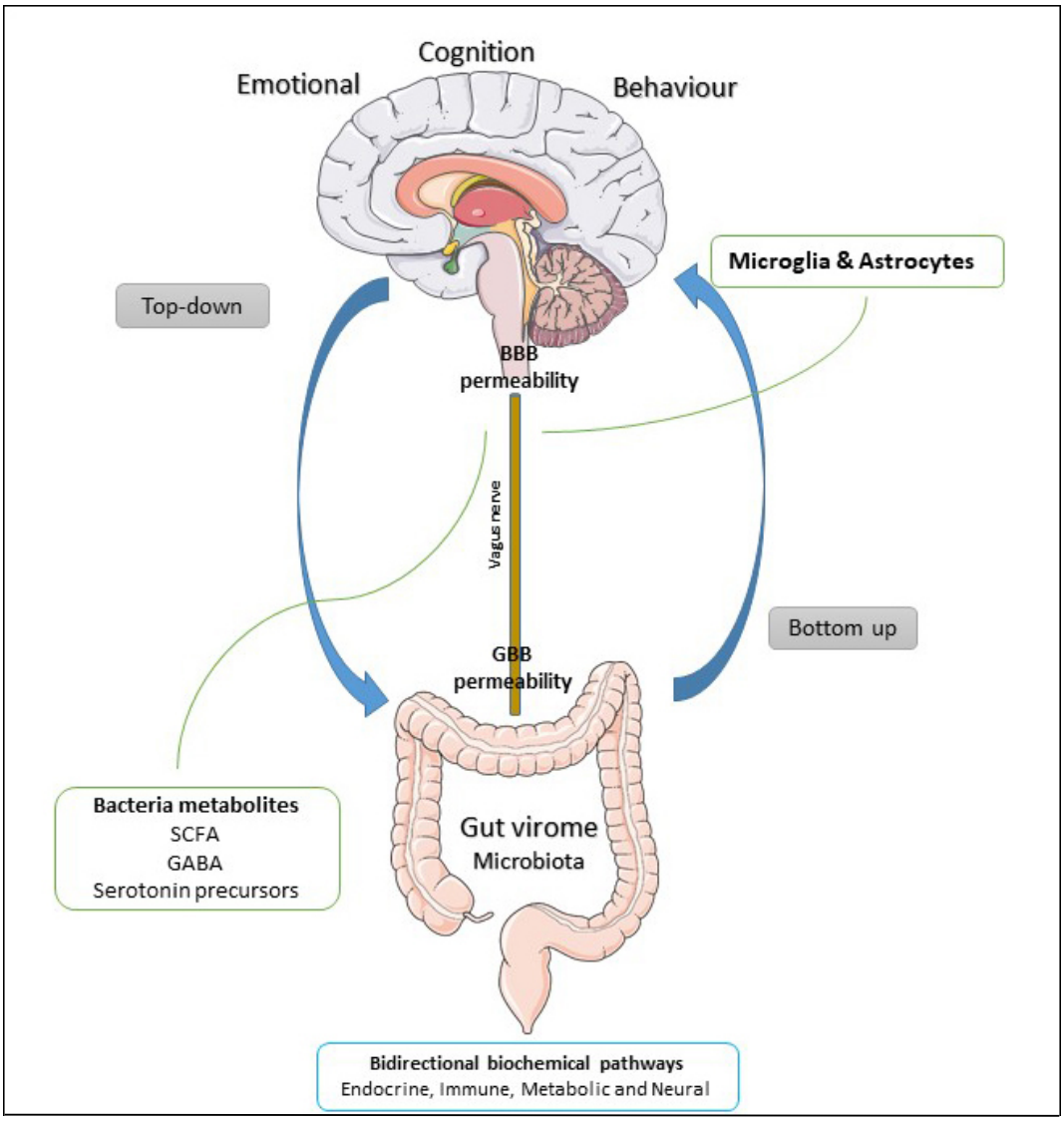


Figure 1: The GUT-BRAIN-AXIS (GBA): The gut microbiome communicates with the Central Nervous System (CNS) predominantly through microbial-derived intermediates, with Short Chain Fatty Acids (SCFAs), secondary Bile Acids (2Bas), and tryptophan metabolites. The GBA can send and receive data messages in both directions: from the brain to the gut and from the gut to the brain.

THE GUT-BRAIN AXIS

The connection between the brain and the intestinal tract is known as the "Gut-Brain Axis" (GBA). The two systems have a strong and reciprocal interaction. The gut and the brain are connected by an information exchange network that encompasses the central nervous, endocrine, metabolic, and immune systems. The GBA has the ability to transfer information in both directions: "top-down" from the brain to the gut and "bottom-up" from the gut to the brain. In addition to the hypothalamic-pituitary-adrenal axis and endocrine

pathways (i.e., intestinal peptides and hormones), there is mounting evidence that bacteria metabolites (e.g. SCFAs, neurotransmitters, and their precursors) affect the levels of related metabolites in the brain via blood circulation, regulating brain functions and cognition. Gut microbiota can also communicate with the brain via the local neurological system (e.g., enteric nerves, vagus nerve), sending very rapid messages to the brain. The gut microbiome communicates with the CNS predominantly through microbial-derived intermediates, with SCFAs, 2BAs, and tryptophan metabolites being the most well-studied examples (*Wikoff* et al., 2009, *Tolhurst* et al., 2012, *Yano* et al., 2015) (Figure 1). However, the interaction between gut viruses, mainly bacteriophages, brain and cognition is fairly unexplored, and only recent studies have shown interactions among the gut phageome, gut bacteriome and cognition (*Mayneris-Perxachs* et al., 2021).

GUT VIROME AND METABOLIC DISEASES

The study of the microbiome and its impact on cognition began with significant discoveries in animal studies (*Braniste* et al., 2014). The gut microbiome is being implicated in a growing number of investigations as a crucial role in the regulation of neurodegenerative processes, cognition modulation, and neurological diseases (*Cryan* et al., 2020; *Morais* et al., 2021).

At the same time, many studies have focused on bacteria (*Morais* et al., 2021). Nevertheless, the gut human viral community has been less studied and recent data reveal that viruses can have a significant impact on physiology and the development of diseases (*Mirzaei* and *Maurice*, 2017; *Keen* and *Dantas*, 2018) through microbiome dysbiosis. There is evidence about the relationship between the human virome and metabolic diseases such as obesity, type 2 diabetes and type 1 diabetes (*Zhao* et al., 2017, *Ma* et al., 2018, *Tetz* et al., 2019, *Vehik* et al., 2019, *Wook* et al., 2019, *Cinek* et al., 2021, *Bikel* et al., 2021, *Hasan* et al., 2021).

Obesity

Alterations of normal microbiota composition are well known to occur in subjects with obesity (*Arnoriaga-Rodríguez* et al., 2020, 2021; *Sandoval-Vargas* et al., 2021). In mice, Bacteria belonging to *Firmicutes* phylum had a positive correlation with total viral content, while *Bacteroidetes* phylum and *Bifidobacterium* genera had a negative correlation with total viral content (*Yadav* et al., 2016). The *Caudovirales* bacteriophages dominated the gut virome (*Biker* et al., 2021, *Yang* et al., 2021). *Escherichia phage, Geobacillus phage*, and *Lactobacillus phage* showed the highest relative abundance among the differential species.

The richness and diversity of the gut virome could vary depending on age and geographic factors. *Bikel* et al., (2021) found that the phage diversity and richness of people with obesity tended to rise in childhood. In contrast, in the adulthood the trend was in the opposite direction: individuals with obesity had lower gut virome richness (Chao1 index) and diversity (Shannon index) than subjects without obesity (*Yang* et al., 2021).

In childhood, the abundance of various phage contigs was linked to gut bacterial taxa as well as anthropometric and biochemical markers in subjects with obesity and metabolic syndrome, including increased serum lipid and glucose levels (*Bikel* et al., 2021). Interestingly, a negative relationship between

BMI, HDL cholesterol and triglyceride levels with the abundance of certain phage contigs was observed.

In comparison with subjects without obesity, 11 virus species were shown to be enriched in subjects with obesity. *Escherichia* phage, *Geobacillus* phage, and *Lactobacillus* phage showed the highest relative abundance among the differential species, showing distinct gut virus abundance and taxonomic compositions (*Yang* et al., 2021).

The gut virome changed after losing weight, either through, diet, exercise or after surgical interventions such gastric bypasses and vertical band gastroplasty (Sandoval-Vargas et al., 2021).

Type 2 Diabetes (T2D)

T2D is a metabolic condition associated with obesity-related insulin resistance with characteristic alterations in the gut microbiota composition (*Larsen* et al., 2010).

Yang et al., (2021) investigated alpha diversity across subjects with and without type 2 diabetes and lean controls. Subjects with obesity and T2DM had a more disrupted gut viral dysbiosis, with reduction in diversity and loss of beneficial viruses, and pathogen conversion of beneficial viruses compared with subjects with obesity alone.

No significant difference was observed in viral Chao1 richness or Shannon's diversity in subjects with obesity without type 2 diabetes when compared with control subjects. On the contrary, subjects with both obesity and T2DM had lower viral richness and diversity and distinct gut viral profiles when compared with lean controls. In comparison to lean controls, 17 viral species were differentially present in subjects with obesity and T2DM. Four viral species (*Micromonas pusilla virus*, *Cellulophaga phage*, *Bacteroides phage*, and *Halovirus*) were found to be elevated in obesity with T2DM, while 13 viral species (*Hokovirus*, *Klosneuvirus*, and *Catovirus*, among others) were found to be decreased. When T2DM patients were compared to patients without T2DM, they found 28 distinct virus species. Finally, these authors also postulated that geographic considerations may be linked to gut virome variation.

Type 1 Diabetes (T1D)

The intestinal microbiota has been linked to the development of autoimmune illnesses, including T1D, according to evidence from murine models. Environmental factors, notably bacteria and viruses, are thought to play a role in the aetiology of T1D (*Faulkner* et al., 2021). Dysbiosis has been linked to disease development in subjects who are at risk for T1D (*Needell* et al., 2016) while SCFAs were increased in patients with T1D. The gut microbiota may play a role in islet destruction (*Brown* et al., 2011). Interestingly, autoimmunity in T1D was associated with changes in bacteriophages and the *Circoviridae* family was linked to a protective effect over autoimmunity. However, the involved mechanisms remain unclear (*Zhao* et al., 2017).

NUTRITION COULD IMPACT THE RELATIONSHIP BETWEEN GUT VIROME AND COGNITION

Recent research has shown that viruses can have a significant impact on the physiology of their bacterial hosts (*Mirzaei* and *Maurice*, 2017; *Keen* and *Dantas*, 2018). Bacteriophages are well known to constitute the most common members of the human virome. Temperate (lysogenic) bacteriophages can

transfer genes to their bacterial hosts, changing their phenotypic and modifying gene expression. Despite this fact, prophages are found in more than 80% of bacterial genomes. As a result, bacteriophages may have a significant impact on bacterial diversity and function, as well as human health (*Mirzaei* and *Maurice*, 2017; *Keen* and *Dantas*, 2018).

A recent study has explored the relationship between gut-resident bacteriophages and the microbiome's structure and metabolism, as well as their effects on cognition (*Mayneris-Perchachs* et al., 2022). The authors found that the presence of, *Caudovirales* bacteriophages in the gut microbiome was associated with improved executive function, specifically, cognitive flexibility and working memory. Specific *Caudovirales* (the former *Siphoviridae* family with the old taxonomy comprising the new *Demerecviridae*, *Drexlerviridae*, and *Siphoviridae* families) levels were positively associated with cognitive flexibility, whilst *Microviridae* counts were negatively associated with this trait.

According to gene and genome analysis of unassembled and assembled data, most of the *Caudovirales* were uncultured and uncharacterized, while others putatively infected predominantly *Lactococcus* spp. and other gut bacteria belonging to *Enterobacteriacaea*, *Firmicutes* (e.g., *Eubacterium rectale*), or *Bacteroidetes*. The gene content and annotation of these *Caudovirales* revealed common gene traits, as those coding for structural proteins (capsid, portal, neck, and tail) and other *Caudovirales* functional proteins (e.g., terminases). For several of these *Caudovirales*, metagenomics assembly resulted in a fragmented genome assembly, particularly for *Lactococcus* viruses, which have been linked to higher performance in central executive processes (*Mayneris-Perchachs* et al., 2022). Within the *Caudovirales*, a strong positive relationship between *Siphoviridae* levels (as per new genome-based taxonomy) and cognitive flexibility was also disclosed. Unlike other *Caudovirales* levels, *Siphoviridae* levels were likewise associated with improved inhibitory control (meaning being less impulsive) and short- and long-term memory, underlining the potential importance of the *Siphoviridae* family in cognitive function.

On the other hand, some the counts of ssDNA *Microviridae* were linked to a worsening of executive function. *Microviridae* levels correlated positively with fat mass, confirming recent findings that showed their rise after a high-fat diet (*Schulfer* et al., 2020). In both unassembled and assembled data, *Microviridae* signature genes and proteins were clearly recognized. Some of them resembled *Escherichia* phage alpha3 and uncultured *Microviridae* seen in the stomach before. Surprisingly, identification of putative hosts revealed that some *Microviridae* infect *Bacteroidetes* (most likely *Alistipes onderdonkii*), and one *Microviridae* virus (contig name c055944) showed a broad host range because CRISPR spacers from *Ruminococcus* spp., *Oscilobacteriales*, and *Lachnospiracea* matched viral protospacers of this virus. *Bacteriophages* may play a crucial role in host health and disease by altering bacterial communities through transposition, induction, and horizontal gene transfer (*Keen* and *Dantas*, 2018). When Mayneris and co-authors (*Mayneris* et al., 2022) looked at the relationships between these bacteriophages and bacterial composition and functionality, Lactic acid bacteria (*Lactobacillales* order), particularly *Streptococcus, Lactobacillus, Lactococcus*, and *Enterococcus* species, were positively associated with specific

Caudovirales levels, while *Bacteroides* species were inversely associated. In fact, all known lactic acid bacteria phages are classified as *Caudovirales*, with the majority of them belonging to the *Siphoviridae* family (*Murphy* et al., 2017). In this study, 40% of the species most associated with certain *Caudovirales* were also associated with cognitive flexibility. *Microviridae* levels, on the other hand, were negatively linked to various *Lactobacillus, Streptococcus*, and *Enterococcus* species, while they were positively linked to *Bacteroides* and *Prevotella* species. In addition, subjects with increased specific *Caudovirales* had better phonemic verbal fluency (specific executive function related to language) and information processing speed (*Mayneris-Perxachs* et al., 2022).

A consistent positive relationship between specific *Caudovirales* levels and *Lactococcus* lactis, as well as several *Lactobacillus* (*L. crispatus, L. plantarum, L. salivarius*, and *Lactobacillus* uc) and *Streptococcus* (*S. mitis, S. salivarius, S. vestibularis, and Streptococcus uc*) species in three out of four cohorts. S. *salivarius* and S. *mitis* are the most common *streptococcal* species in human milk microbiota (*Martin* et al., 2016), whereas *L. lactis* and *Lactobacillus* sp. are commonly employed in dairy product fermentation (*Murphy* et al., 2017). In the human milk microbiota, *S. salivarius* and *S. mitis* are the most common *streptococcal* species (*Martin* et al., 2016). They identified consistent positive relationships between *Caudovirales* levels and the intake of dairy products, as well as with the plasma levels of medium-chain fatty acids, naturally prevalent in dairy fat. The authors also objectived certain *Caudovirales*-linked lactic acid bacteria and dairy products. The *Microviridae* family, on the other hand, showed a negative relationship with medium-chain fatty acids. In this context, it is interesting to mention that the supplementation of mice and humans with medium-chain fatty acids has been demonstrated to promote synaptic plasticity and cognitive performance (*Page* et al., 2009; *Wang* and *Mitchell*, 2016).

Significant correlations between bacterial pathways, bacteriophages, and human host executive functions were also discovered using functional analyses based on the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway. *Caudovirales* levels were found to be substantially linked to folate-mediated one-carbon metabolism (*Mayneris-Perxachs* et al., 2022). Folate metabolism is important for a variety of physiological activities because it provides the 1C units needed for cellular operations (*Ducker* and *Rabinowitz*, 2017). It is also a part of the methionine cycle, which is required for the production of S-adenosylmethionine (SAM), the universal methyl donor in a variety of methylation processes, including DNA methylation. It modulates redox defence by generating antioxidants like taurine and glutathione from cysteine via the trans-sulfuration mechanism. All of these bacterial pathways were positively linked to specific *Caudovirales* levels and negatively with *Microviridae* levels. A link between the metabolism of vitamins B2 and B6 (important cofactors in the folate cycle) and the presence of some *Caudovirales* was also uncovered. The bacterial genes *thyX* and *dut*, both involved in folate-mediated pyrimidine biosynthesis, had the strongest relationships with *Caudovirales* levels, according to functional analyses at the enzyme level. Thymidylate synthase is encoded by the *thyX* gene (*TYMS* in humans). Reduced *TYMS* expression is known to cause a misalignment of DNA synthesis and methylation, which is crucial for neurodevelopment, synaptic plasticity, and

memory (*Heyward* and *Sweatt*, 2015). In addition, a lack of folate-mediated one-carbon metabolism has been linked to neurodegenerative illnesses, which could be caused by a lack of dTMP synthesis and subsequent uracil misincorporation into DNA (*Blount* et al., 1997; *Ducker* and *Rabinowitz*, 2017). Other critical pathways in the central nervous system, such as glutamatergic, GABAergic, dopaminergic, serotonergic synapse, and retrograde endocannabinoid transmission, were similarly negatively related with certain *Caudovirales* levels. Other closely connected bacterial genes were found to be involved in folate-mediated histidine catabolism *(FTCD, FTCD*) and purine biosynthesis (*FTCD, FTCD*) (*purH, purU*) (*Mayneris-Perxachs* et al., 2022).

Finally, many circulating and faecal metabolites were also associated with *Microviridae* and *Caudovirales* levels. Most of these metabolites were directly implicated in one-carbon metabolism: choline, glycine, formate, histidine, and glucose are among the metabolites that feed 1C units to the folate pool, as are related catabolites (urocanate, glutamate, inosine, β-aminoisobutyric acid, and methionine sulfoxide). The most important sources of folate 1C units, choline and glycine (*Ducker* and *Rabinowitz*, 2017), exhibited the closest relationships with *Microviridae* and specific *Caudovirales* levels. Closing the circle, bacterial metabolic pathways for glycine and histidine were also linked to particular *Caudovirales* and *Microviridae* levels. Glycine is produced by the breakdown of dietary choline and serine, which provide carbon units to the 1C-metabolism. Serine can also be made from 3-phosphorylglycerate, which is a glycolysis intermediate. The glycine cleavage system (GCS), which produces a carbon unit for the methylation of tetrahydrofolate, is also a 1C source (THF). Bacteriophage levels were consistently linked to genes involved in the GCS (*gcvH, gcvP, and gcvR*), serine synthesis (serB and serA), and choline transport and catabolism (*serB and serA*) (*sox and opuD*). The GCS transcriptional repressor (gcvR) was associated with the Microviridae family (*Mayneris-Perxachs* et al., 2022), whereas GCS genes exhibited the largest negative correlation with particular *Caudovirales* levels. In both mice and humans, mutations in genes encoding the GCS have been demonstrated to lead to neural tube abnormalities and neurological dysfunction (*Kure* et al., 2006; *Narisawa* et al., 2012).

In order to validate the findings, the authors performed a microbiota transplantation from humans to mice. A dose-response effect based on the specific *Caudovirales* levels in the donor's microbiome was found 4 weeks later: the higher the *Caudovirales* levels, the higher the scores in the novel object recognition test, which is used to assess cognition, particularly immediate memory. Increased *Microviridae* levels in the donor's microbiome, on the other hand, were linked to recipient mice's cognitive impairment. They also investigated whether faecal microbiota transplantation had an effect on the transcriptome of the recipient's prefrontal cortex of mice, involved in executive processes and memory. Of note, 23 and 18 genes were up- and down-regulated, respectively, in response to the donor's specific *Caudovirales* levels, according to RNA sequencing. *Microviridae* levels in donors were linked to up- and down-regulation of 18 and 10 genes, respectively (*Mayneris-Perxachs* et al., 2022) Several of the most up-regulated gene transcripts with increased donor's specific *Caudovirales* levels were well known memory-promoting genes (e.g., *Arc, Fos, Egr2,* and *Btg2)*, whereas those down-regulated (*Ide* and

Ppp1r42) were memory suppressors (*Poon* et al., 2020).

Based on gene ontology analysis, cognition was identified as the most over-represented biological function linked to the donor's unique *Caudovirales* levels (*Mayneris-Perxachs* et al., 2022). In the hippocampus and retrosplenial cortex of adult mice, learning and memory acquisition is known to lead to increased expression of the *IEGs Arc, Fos, Btg2, Sik1, Dusp1, Ier2, and Egr2* (*Peixoto* et al., 2015).

Finally, in this study, the exposure of *Drosophila melanogaster* to lactococcal 936-type bacteriophages led to improved memory retention, changing the expression of memory-related genes in the brain. These findings revealed that thermolabile components in whey powder, including the presence of bacteriophages in this product, could improve memory.

An old paper published in Nature in 1971 showed "*bacterial virus gene expression in human cells*" (*Merrill* et al., 1971). Bacteriophages have long been known to be able to cross the blood-brain barrier (*Frenkel* and *Solomon*, 2002). This raises the question as to whether intrabody bacteriophages can accumulate within the central nervous system or brain, and mediate direct behavioural and neurological effects in mammals. As noted in a seminal paper by Dr Jeremy Barr in 2017, "*practically no research has been done investigating the role and function of native intrabody bacteriophages on the central nervous system and brain*" (*Barr*, 2017). As bacteriophages had been described to bind to β-amyloid and α-synuclein, it was hypothesised that "*it is possible that bacteriophages act as cleaners of the brain" and that "we must consider the possibility of bacteriophage mind control*" (*Barr*, 2017).

Recent findings seem to go far beyond of initial expectations. Not only *Siphoviridae* and *Microviridae* counts were reciprocally associated with executive function (one of the domains of cognition) in two independent cohorts, but also with a specific microbiome profile in four independent cohorts. It was also noted that gut bacterial functions and plasma and faecal metabolites run in parallel to bacteriophage counts, integrated in a network impacting cognition. A kind of dose-response effects of bacteriophages was observed in the human gut microbiome transplanted to mice: the genes that most changed in recipient mice were precisely those involved in memory in a concordant manner with mice cognition (*Mayneris-Perxachs* et al., 2022).

In summary, there is little doubt that the novel findings reported so far are promising to decipher new therapeutic targets through diet and nutrition, focusing on the microbiota and its relationships with body systems. The impact on the central nervous system through treatments for cognitive and memory impairment could include the use of known bacteriophages.

ACKNOWLEDGEMENTS

We acknowledge the help of Irene Navarro Uclés in the edition and corrections of this manuscript.

LITERATURE

American Psychiatric Association: The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, Text Revision (DSM-5-TR™). American Psychiatric Association Publishing, Inc. Washington, DC, USA (2013).

Arnoriaga-Rodríguez, M. and Fernández-Real, J.M.: Microbiota impacts on chronic inflammation and metabolic syndrome - related cognitive dysfunction. Rev. Endocr. Metab. Disord. 20, 473-480 (2019).

Arnoriaga-Rodríguez, M., Mayneris-Perxachs, J., Burokas, A., Contreras-Rodríguez, O., Blasco, G., Coll, C., Biarnés, C., Miranda-Olivos, R., Latorre, J., Moreno-Navarrete, J.M., Castells-Nobau, A., Sabater, M., Palomo-Buitrago, M.E., Puig, J., Pedraza, S., Gich, J., Pérez-Brocal, V., Ricart, W., Moya, A., Fernández-Real, X., Ramió-Torrentà, L., Pamplona, R., Sol, J., Jové, M., Portero-Otin, M., Maldonado, R., and Fernández-Real, J.M.: Obesity Impairs Short-Term and Working Memory through Gut Microbial Metabolism of Aromatic Amino Acids. Cell Metab. 32, 548-560 (2020).

Arnoriaga-Rodríguez, M., Mayneris-Perxachs, J., Contreras-Rodríguez, O., Burokas, A., Ortega-Sanchez, J.A., Blasco, G., Coll, C., Biarnés, C., Castells-Nobau, A., Puig, J., Garre-Olmo, J., Ramos, R., Pedraza, S., Brugada, R., Vilanova, J.C., Serena, J., Barretina, J., Gich, J., Pérez-Brocal, V., Moya, A., Fernández-Real, X., Ramio-Torrentà, L., Pamplona, R., Sol, J., Jové, M., Ricart, W., Portero-Otin, M., Maldonado, R., and Fernández-Real, J.M.: Obesity-associated deficits in inhibitory control are phenocopied to mice through gut microbiota changes in one-carbon and aromatic amino acids metabolic pathways. Gut 70, 2283-2296 (2021).

Barr, J.J.: A bacteriophages journey through the human body. Immunol. Rev. 279, 106-122 (2017).

Bikel, S, López-Leal, G, Cornejo-Granados, F, Gallardo-Becerra, L., García-López, R., Sánchez, F., Equihua-Medina, E., Ochoa-Romo, J.P., López-Contreras, B.E., Canizales-Quinteros, S., Hernández-Reyna, A., Mendoza-Vargas, A., and Ochoa-Leyva, A.: Gut dsDNA virome shows diversity and richness alterations associated with childhood obesity and metabolic syndrome. iScience 24, 102900 (2021).

Blount, B.C., Mack, M.M., Wehr, C.M., MacGregor, J.T., Hiatt, R.A., Wang, G., Wickramasinghe, S.N., Everson, R.B., and Ames, B.N.: Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. Proc. Natl. Acad. Sci. USA 94, 3290-3295 (1997).

Braniste, V., Al-Asmakh, M., Kowal, C., Anuar, F., Abbaspour, A., Tóth, M., Korecka, A., Bakocevic, N., Ng, L.G., Kundu, P., Gulyás, B., Halldin, C., Hultenby, K., Nilsson, H., Hebert, H., Volpe, B.T., Diamond, B., and Pettersson, S.: The gut microbiota influences blood-brain barrier permeability in mice. Sci. Transl. Med. 19, 263 (2014).

Brown, C.T., Davis-Richardson, A.G., Giongo, A., Gano, K.A., Crabb, D.B., Mukherjee, N., Casella, G., Drew, J.C., Ilonen, J., Knip, M., Hyöty, H., Veijola, R., Simell, T., Simell, O., Neu, J., Wasserfall, C.H., Schatz, D., Atkinson, M.A., and Triplett, E.W.: Gut microbiome metagenomics analysis suggests a functional model for the development of autoimmunity for type 1 diabetes. PLoS One 6, e25792 (2011).

Chen, Y., Xu, J., and Chen, Y.: Regulation of Neurotransmitters by the Gut Microbiota and Effects on Cognition in Neurological Disorders. Nutrients 13, 2099 (2021).

Cinek, O., Kramna, L., Odeh, R., Alassaf, A., Ibekwe, M.A.U., Ahmadov, G., Elmahi, B.M.E., Mekki, H., Lebl, J., and Abdullah, M.A.: Eukaryotic viruses in the fecal virome at the onset of type 1 diabetes: A study from four geographically distant African and Asian countries. Pediatr. Diabetes 22, 558-566 (2021).

Cryan, J.F., O'Riordan, K.J., Sandhu, K.,

Peterson, V., and Dinan, T.G.: The gut microbiome in neurological disorders. Lancet Neurol. 19, 179-194 (2020).

Ducker, G.S. and Rabinowitz, J.D.: One-carbon metabolism in health and disease. Cell Metab. 25, 27-42 (2017).

Durgan, D.J., Lee, J., McCullough, L.D., and Bryan, Jr., R.M.: Examining the Role of the Microbiota-Gut-Brain Axis in Stroke. Stroke 50, 2270-2277 (2019).

Faulkner, C.L., Luo, Y.X., Isaacs, S., Rawlinson, W.D., Craig, M.E., and Kim, K.W.: The virome in early life and childhood and development of islet autoimmunity and type 1 diabetes: A systematic review and meta-analysis of observational studies. Rev. Med. Virol. 31, 1-14 (2020).

Fava, F., Rizzetto, L., and Tuohy, K.M.: Gut microbiota and health: connecting actors across the metabolic system. Proc. Nutr. Soc. 78, 177-188 (2019).

Heyward, F.D. and Sweatt, J.D.: DNA Methylation in Memory Formation: Emerging Insights. Neuroscientist 21, 475-489 (2015).

Frenkel, D. and Solomon, B.: Filamentous phage as vector-mediated antibody delivery to the brain. Proc. Natl. Acad. Sci. USA 99, 5675-5679 (2002).

Hooper, L.V., Littman, D.R., and Macpherson, A.J.: Interactions between the microbiota and the immune system. Science 336, 1268-1273 (2012).

Jones, B.V., Begley, M., Hill, C., Gahan, C.G., and Marchesi, J R.: Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome. Proc. Natl. Acad. Sci. USA 105, 13580-13585 (2008).

Keen, E.C. and Dantas, G.: Close Encounters of Three Kinds: Bacteriophages, Commensal Bacteria, and Host Immunity. Trends Microbiol. 26, 943-954 (2018).

Koh, A., De Vadder, F., Kovatcheva-Datchary, P., and Backhed, F.: From dietary fiber to host physiology: short-chain fatty acids as key bacterial metabolites. Cell 165, 1332-1345 (2016).

Kure, S., Kato, K., Dinopoulos, A., Gail, C., DeGrauw, T.J., Christodoulou, J., Bzduch, V., Kalmanchey, R., Fekete, G., Trojovsky, A., Plecko, B., Breningstall, G., Tohyama, J., Aoki, Y., and Matsubara, Y.: Comprehensive mutation analysis of GLDC, AMT, and GCSH in nonketotic hyperglycinemia. Hum. Mutat. 27, 343-352 (2006).

Latorre, R., Sternini, C., De Giorgio, R., and Greenwood-Van Meerveld, B.: Enteroendocrine cells: a review of their role in brain-gut communication. Neurogastroenterol. Motil. 28, 620-630 (2016).

Ma, Y., You, X., Mai, G., Tokuyasu, T., and Liu, C.: A human gut phage catalog correlates the gut phageome with type 2 diabetes. Microbiome 6, 24 (2018).

Mahony, J., McDonnell, B., Casey, E., and van Sinderen, D.: Phage-Host Interactions of Cheese-Making Lactic Acid Bacteria. Annu. Rev. Food Sci. Technol. 7, 267-285 (2016).

Martín, V., Mediano, P., Del Campo, R., Rodríguez, J.M., and Marín, M.: Streptococcal diversity of human milk and comparison of different methods for the taxonomic identification of streptococci. J. Hum. Lact. 32, NP84–NP94 (2016).

Mayneris-Perxachs, J., Castells-Nobau, A., Arnoriaga-Rodríguez, M., Garre-Olmo, J., Puig, J., Ramos, R., Martínez-Hernández, F., Burokas, A., Coll, C., Moreno-Navarrete, J.M., Zapata-Tona, C., Pedraza, S., Pérez-Brocal, V., Ramió-Torrentà, L., Ricart, W., Moya, A., Martínez-García, M., Maldonado, R., and Fernández-Real, J.M.: *Caudovirales* bacteriophages are associated with improved executive function and memory in flies, mice, and humans. Cell Host Microbe 30, 340-356 (2022).

Mentch, S.J., Mehrmohamadi, M., Huang, L., Liu, X., Gupta, D., Mattocks, D., Gómez Padilla, P., Ables, G., Bamman, M.M., Thalacker-Mercer, A.E., Nichenametla, S.N., and Locasale, J.W.: Histone Methylation Dynamics and Gene Regulation Occur through the Sensing of One-Carbon Metabolism. Cell Metab. 22, 861-73 (2015).

Merril, C.R., Geier, M.R., and Petricciani, J.C.: Bacterial virus gene expression in human cells. Nature 233, 398-400 (1971).

Mirzaei, M.K. and Maurice, C.F.: Ménage à

trois in the human gut: interactions between host, bacteria and phages. Nat. Rev. Microbiol. 15, 397-408 (2017).

Morais, L.H., Schreiber, H.L. and Mazmanian, S.K.: The gut microbiota–brain axis in behaviour and brain disorders. Nat. Rev. Microbiol. 19, 241–255 (2021).

Narisawa, A., Komatsuzaki, S., Kikuchi, A., Niihori, T., Aoki, Y., Fujiwara, K., Tanemura, M., Hata, A., Suzuki, Y., Relton, C.L., Grinham, J., Leung, K.-Y., Partridge, D., Robinson, A., Stone, V., Gustavsson, P., Stanier, P., Copp, A.J., Greene, N.D.E., Tominaga, T., Matsubara, Y., and Kure, S.: Mutations in genes encoding the glycine cleavage system predispose to neural tube defects in mice and humans. Hum. Mol. Genet. 21, 1496-1503 (2012).

Needell, J.C. and Zipris, D.: The Role of the Intestinal Microbiome in Type 1 Diabetes Pathogenesis. Curr. Diab. Rep. 16, 89 (2016).

O'mahony, S.M., Clarke, G., Borre, Y.E., Dinan, T.G., and Cryan, J.F.: Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. Behav. Brain Res. 277, 32-48 (2015).

Page, K.A., Williamson, A., Yu, N., McNay, E.C., Dzuira, J., McCrimmon, R.J., and Sherwin, R.S.: Medium-chain fatty acids improve cognitive function in intensively treated type 1 diabetic patients and support in vitro synaptic transmission during acute hypoglycemia. Diabetes 58, 1237-1244 (2009).

Peixoto, L.L., Wimmer, M.E., Poplawski, S.G., Tudor, J.C., Kenworthy, C.A., Liu, S., Mizuno, K., Garcia, B.A., Zhang, N.R., Giese, K., and Abel, T.: Memory acquisition and retrieval impact different epigenetic processes that regulate gene expression. BMC Genomics. 16, Suppl. 5, S5 (2015).

Sandoval-Vargas, D., Concha-Rubio, N.D., Navarrete, P., Castro, M., and Medina, D.A.: Short Communication: Obesity Intervention Resulting in Significant Changes in the Human Gut Viral Composition. Appl. Sci. 11, 10039 (2021).

Schulfer, A., Santiago-Rodriguez, T.M., Ly, M., Borin, J.M., Chopyk, J., Blaser, M.J., and Pride, D.T.: Fecal Viral Community Responses to High-Fat Diet in Mice. mSphere 5, e00833-19 (2020).

Socała, K., Doboszewska, U., Szopa, A., Serefko, A., Włodarczyk, M., Zielińska, A., Poleszak, E., Fichna, J., and Wlaź, P.: The role of microbiota-gut-brain axis in neuropsychiatric and neurological disorders. Pharmacol. Res. 172, 105840 (2021).

Tetz, G., Brown, S.M., Hao, Y., and Tetz, V.: Type 1 Diabetes: An Association Between Autoimmunity, the Dynamics of Gut Amyloidproducing E. coli and Their Phages. Sci. Rep. 9, 9685 (2019).

Tolhurst, G., Heffron, H., Lam, Y.S., Parker, H.E., Habib, A.M., Diakogiannaki, E., Cameron, J., Grosse, J., Reimann, F., and Gribble, F.M.: Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein-coupled receptor FFAR2. Diabetes. 61, 364-371 (2012).

Topping, D.L. and Clifton, P.M.: Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiol. Rev. 81, 1031-1064. (2001).

Vehik, K., Lynch, K.F., Wong, M.C., Tian, X., Ross, M.C., Gibbs, R.A., Ajami, N.J., Petrosino, J.F., Rewers, M., Toppari, J., Ziegler, A.G., She, J.X., Lernmark, A., Akolkar, B., Hagopian, W.A., Schatz, D.A., Krische,r J.P., Hyöty, H., Lloyd, R.E.; TEDDY Study Group: Prospective virome analyses in young children at increased genetic risk for type 1 diabetes. Nat. Med. 25, 1865-1872 (2019).

Wang, D. and Mitchell, E.S.: Cognition and Synaptic-Plasticity Related Changes in Aged Rats Supplemented with 8- and 10-Carbon Medium Chain Triglycerides. PLoS One 11, e0160159 (2016).

Wikoff, W.R., Anfora, A.T., Liu, J., Schultz, P.G., Lesley, S.A., Peters, E.C., and Siuzdak, G.: Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proc. Natl. Acad. Sci. USA. 106, 3698-3703 (2009).

Wook, K.K., Allen, D.W., Briese, T., Couper, J.J., Barry, S.C., Colman, P.G., Cotterill, A.M., Davis, E.A., Giles, L.C., Harrison, L.C., Harris, M., Haynes, A., Horton, J.L., Isaacs, S.R., Jain, K., Lipkin, W.I., Morahan, G., Morbey, C., Pang, I.C.N., Papenfuss, A.T., Penno, M.A.S., Sinnott, R.O., Soldatos, G., Thomson, R.L., Vuillermin, P.J., Wentworth. J.M., Wilkins, M.R., Rawlinson, W.D., Craig, M.E.; ENDIA Study Group: Distinct Gut Virome Profile of Pregnant Women with Type 1 Diabetes in the ENDIA Study. Open Forum Infect. Dis. 6, ofz025 (2019).

World Health Organization. Obesity and overweight. Accessed May 10 (2019) https://www.who.int/newsroom/fact-sheets/detail/obesity-and-overweight.

Yadav, H., Jain, S., Nagpal, R., and Marotta, F.: Increased fecal viral content associated with obesity in mice. World J. Diabetes 7, 316-320 (2016).

Yang, K., Niu, J., Zuo, T., Sun, Y., Xu, Z., Tang, W., Liu, Q., Zhang, J., Ng, E.K.W., Wong, S.K.H., Yeoh, Y.K., Chan, P.K.S., Chan, F.K.L., Miao, Y., Ng, S.C.: Alterations in the Gut Virome in Obesity and Type 2 Diabetes Mellitus. Gastroenterology 161, 1257-1269 (2021).

Yano, J.M., Yu, K., Donaldson, G.P., Shastri, G.G., Ann, P., Ma, L., Nagler, C.R., Ismagilov, R.F., Mazmanian, S.K., and Hsiao, E.Y.: Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. Cell 161, 264-276 (2015).

Zhao, G., Vatanen, T., Droit, L., Park, A., Kostic, A.D., Poon, T.W., Vlamakis, H., Siljander, H., Härkönen, T., Hämäläinen, A.M., Peet, A., Tillmann, V., Ilonen, J., Wang, D., Knip, M., Xavier, R.J., and Virgin, H.W.: Intestinal virome changes precede autoimmunity in type I diabetes-susceptible children. Proc. Natl. Acad. Sci. USA 114, E6166-E6175 (2017).

TURNING *IN SILICO* PHAGE INTO *IN VIVO* PHAGE: THE CRASSPHAGE STORY

CIARA A. TOBIN, ANDREY N. SHKOPOROV, and COLIN HILL

APC Microbiome Ireland, School of Microbiology, University College Cork, College Road, Cork T12 K8AF, Ireland

SUMMARY

CrAss-like phages were originally identified as highly abundant and ubiquitous members of the gut microbiome from studies involving metagenomic sequencing and cross-assembly of reads from multiple individuals (**crAss** - **Cr**oss **Ass**embly). More recently, it has been established that crAss-like phages infect bacteria of the phylum Bacteroidetes. One of the most interesting facets to crAss-like phages is their ability to persist at high numbers over extended periods in the laboratory and in the gut without significantly impacting the abundance of their bacterial hosts. Here we recount what is known about crAss-like phages so far, from their *in silico* discovery in 2014 and the subsequent identification of unique genomic features, to the first isolation of crAss001 and the elucidation of various biological characteristics resulting from the study of phage-host pairs *in vivo*. A large amount of information has been gleaned in a relatively short period of time but it is clear that crAss-like phage research is still in its infancy. Future research lies in further *in vivo* work with phage-host pairs, coupled with the isolation of further crAss-like phage from more divergent groups.

INTRODUCTION

CrAss-like phages are an intriguing member of the human gut microbiome. They are both extremely prolific and widespread, and account for over 86% of gut viral genomes. (*Yutin* et al., 2021) They have been identified in faecal samples from all over the globe, and are found in all age groups from infants to the elderly (*Edwards* et al., 2019). While crAssphages are rarely a component of the new-born microbiome, they become increasingly prevalent within the first year of life. It has been suggested that vertical transmission leads to this initial colonisation (*McCann* et al., 2018; *Siranosian* et al., 2020). They have also been shown to be transferred and to stably engraft in recipients of faecal microbiota transplants (*Draper* et al., 2018). CrAss-like phages have been identified in both remote and traditional hunter-gatherer populations, albeit at a reduced prevalence to that of industrialised populations (*Edwards* et al., 2019; *Honap* et al., 2020). CrAss-like phages have also been identified in sequences recovered from old and new world primates, suggesting an ancient association of crAss-like phages with these primates (*Edwards* et al., 2019). CrAss-like phages are generally viewed as benign viruses of the gut. Correlations between crAss-like phages and a number of different health and disease factors have been examined, but no significant associations have been identified (*Honap* et

al., 2020). At the same time, one crAss-like phage, termed IAS virus, was identified in stool samples from HIV-1 patients with unexplained diarrhoea. While no correlations between IAS virus and diarrhoea were established, it was significantly associated with advanced stages of HIV-1 disease (*Oude Munnink* et al., 2014).

IN SILICO DISCOVERY

The founding member of the CrAss-like phages was originally identified *in silico* in 2014 (*Dutilh* et al., 2014). Despite being both highly abundant and ubiquitous, its divergence from other bacteriophage sequences in viral databases allowed it to remain a component of the 'viral dark matter' for decades. Identification of the ~97 Kb dsDNA genome of this prototypical crAssphage (p-crAssphage) came from the re-analysis of publicly available viral metagenomes using cross-assembly software (crAss), from which crAssphage derives its name. A reference independent method, crAss cross-assembles all reads from input samples and counts the number of shared contigs (*Dutilh* et al., 2012). At the time of assembly, fewer than 20 of the 80 predicted ORF's could be assigned a function. While the phage had never been grown in the laboratory, CRISPR spacers and co-occurrence analysis predicted that *Bacteroides* was the likely host of this phage.

Subsequent studies identified variations to the p-crAssphage genome (*Manrique* et al., 2016; *Liang* et al., 2016). This was expanded on by Yutin and colleagues (*Yutin* et al*.,* 2018) in which the use of sensitive protein analysis methods revealed several hundreds of putative members of a wider crAss-like family, found not only in the human gut but also in diverse environments such as groundwater, marine sediments and hypersaline brine. Annotation of a number of putative tail proteins with homologs to phage P22 led to the prediction that crAss-like phages encode for short tails, typical of that of *Podoviridae.* This was later supported by transmission electron microscopy (TEM) images of a faecal filtrate rich in crAss-like phages that was dominated by podoviridae (*Guerin* et al., 2018).

CURRENT CLASSIFICATIONS AND GENERAL GENOMIC FEATURES

Hundreds of additional CrAss-like phage genomes (each assembled from sequences derived from a single individual) have been further classified into four subfamilies (Alphacrassvirinae, Betacrassvirinae, Gammacrassvirinae and Deltacrassvirinae) and ten genera (I - X), based on the percentage of protein coding genes shared between phages. CrAss-like phages sharing > 40% of protein coding genes were classified at the genus level, while CrAss-like phages sharing 20-40% were classified at the subfamily level. (*Guerin* et al., 2018) Recent analysis by Yutin and colleagues (*Yutin* et al., 2021) identified almost 600 crAss-like genomes and expanded classification to include two more groups, epsilon and zeta. It has been proposed that crAss-like phages could form an order within the class Caudoviricetes, with 6 families, comprising the existing 4 subfamilies and the additions of epsilon and zeta (*Walker* et al., 2021).

In general, crAss-like phages have a genome size of ~100 Kb, although they can range from as large as ~150 - 200

Kb in the case of groups epsilon and zeta. Their genomes are divided roughly in two modules, with opposite gene orientation. One side harbours genes responsible for replication and nucleotide metabolism while the other encodes for structural genes (*Yutin* et al., 2018; *Shkoporov* et al., 2018; *Guerin* et al., 2021). At the junction of the two opposite modules 2-3 genes encode a very large RNA polymerase (RNAP) of up to 6000 amino acids in total. The genes encoding these subunits are sometimes on opposite coding strands. These subunits are highly divergent both from each other and other known RNAP proteins. They have been confirmed to be structurally most similar to eukaryotic RNAP that are involved in RNA interference (*Drobysheva* et al., 2020). Analysis of the first crAssphage to have been grown in culture confirms that this large RNAP is a component of the virion and are almost certainly involved in transcription of early phage genes (*Shkoporov* et al., 2018; *Drobysheva* et al., 2020). Why crAss-like phage encode such abnormally large genes for these subunits is unclear.

In addition to these large RNAP genes, crAss-like phages possess a number of other unusual genomic features. Most encode a DNA polymerase from either the A or B family and switching can occur between the two, even within the same subfamily (*Yutin* et al., 2018, 2021). A large number of crAss-like phages, mainly from the beta, zeta and epsilon groups, appear to use alternative genetic codes (*Guerin* et al., 2018; *Yutin* et al., 2021). Typically, this is by the reassignment of stop codon TAG to glutamine or TGA to tryptophan and seems to mainly occur in the late phage genes. Some crAss-like phages, particularly of the zeta group, are characterised by an abnormally high density of introns and inteins *(Yutin* et al., 2021). Whether this spread of mobile genetic elements is uncontrollable due to some aspect of the phage lifestyle or is an adaptive approach used by the phage is so far unknown. Those crAss-like phages isolated to date, p-crAssphage and others used in comparative studies have lacked these features, leading to relatively simple annotation.

IN VITRO ISOLATION AND BIOLOGICAL PROPERTIES

To date, five crAss-like phages have been isolated with their hosts, ΦCrAss001, ΦCrAss002, DAC15, DAC17 and Φ14:2, which infect hosts *Bacteroides intestinalis, Bacteroides xylanisolvens, Bacteroides thetaiotaomicron* and *Cellulophaga baltica* respectively, confirming the previous predictions of *Bacteroidetes* as a host (*Shkoporov* et al., 2018; *Hryckowian* et al., 2020; *Guerin* et al., 2021; *Holmfeldt* et al., 2013). ΦCrAss001 and its closely related phages DAC15 and DAC17 all belong to the subfamily Betacrassvirinae, while ΦCrAss002 is more closely related to p-crAssphage, as it falls into the category of Alphacrassvirinae. Φ14:2 is a distantly related crAss-like phage, isolated from seawater. Our limited knowledge of the biological properties of crAss-like phages comes from the studies of these phage-host pairs.

CrAss-like phages appear to establish an equilibrium with their hosts in liquid culture, in which they can propagate stably over many subcultures, without causing lysis of their hosts. This has also been demonstrated in a mouse model, where CrAss001 can maintain total counts between approximately 10^6-10^8 pfu/mL over a period of 136 days, without causing a reduction in

B. intestinalis counts (*Shkoporov* et al., 2021). This corresponds with a longitudinal study of ten human volunteers, where crAss-like phages were able to stably persist within individuals over a period of one year (*Shkoporov* et al., 2019). The Bacteroides hosts typically encode a number of alternative capsular polysaccharides (CPS) within their genomes, some which seem to be permissive to phage infection, while others are likely to be neutral or protective (*Porter* et al., 2020). Phase variation of these CPS's appear to help to maintain the bacterial population in the presence of phages by creating a resistant subpopulation. The switch to a resistant subtype can occur even in the absence of phages (*Shkoporov* et al., 2021). DAC15 and DAC17 can infect the wild type version of *B. thetaiotaomicron,* which produces 8 different CPS's. On the wild type strain they produce hazy plaques and can be maintained in liquid culture at 10^7-10^8 pfu/mL. When infecting an engineered strain that produces only CPS3, clear plaques are formed and phage counts are maintained at a level one log higher in liquid culture (*Shkoporov* et al., 2021). Interestingly, lysis of the cell culture still does not occur, suggesting that other mechanisms, such as phase variation of outer membrane lipoproteins, may also contribute to the dynamic equilibrium between both partners (*Porter* et al., 2020).

A second mechanism, likely to be working in parallel to phase variation, is an apparent delayed release of phage progeny from infected cells. In classic one step growth curves at an MOI of 1, ΦCrAss001, DAC15 and DAC17 all generate very small burst sizes of between 2.5-8 pfu per infected cell (*Shkoporov* et al., 2018, 2021). In the case of ΦcrAss001, it has been observed that a second small burst occurs around 90 minutes after the first. Despite this, more than 50 phage particles per cell can be observed in TEM's of ΦCrAss001 spotted onto lawns of *B. intestinalis*, and at least 20 copies of ΦCrAss001 genome per copy of the *B. intestinalis* genome are produced within 90 minutes of infection. Intriguingly, this mechanism of delayed release can be overcome by increasing the MOI in *B. theta* but the same cannot be observed for *B. intestinalis* (*Shkoporov* et al., 2021).

While ΦCrAss001, DAC15 and DAC17 have all been demonstrated to behave quite similarly, ΦCrAss002 has a number of additional interesting properties. It is unable to form plaques on its host and can only form opaque spots that are barely visible when a highly concentrated phage stock is spotted onto a lawn of its sensitive bacterial host. Furthermore, propagation in liquid culture only occurs after a minimum of 3 days subculturing and it appears that it is *B. xylanisolvens* that adapts to allow phage infection. Why adaptation by the host is delayed is unknown. Phase variation is a possible contributor here too, with *B. xylanisolvens* possessing many recombination hotspots (*Guerin* et al., 2021).

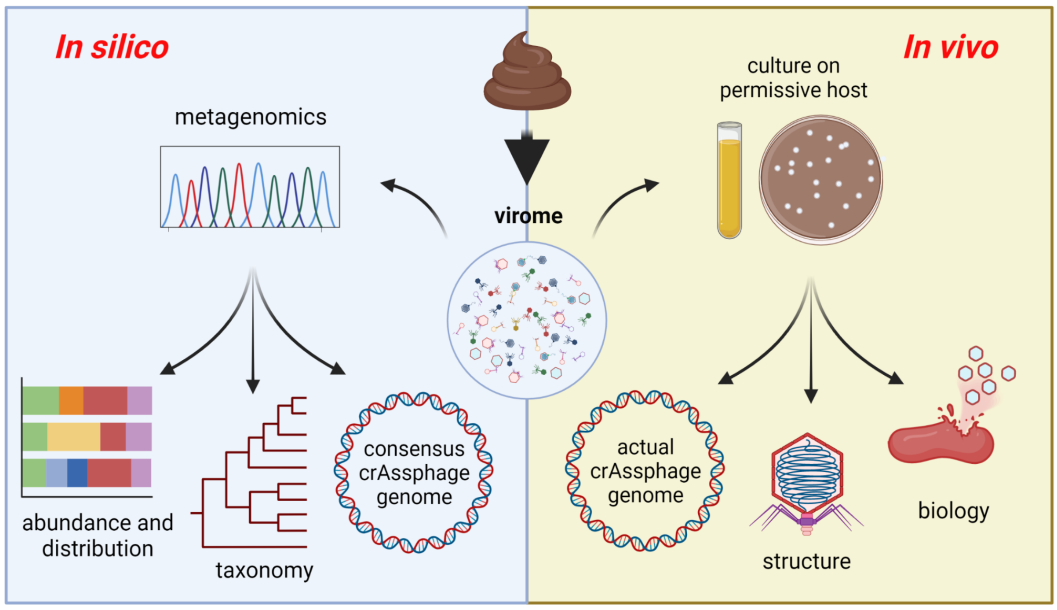


Figure 1: Both *in silico* and *in vivo* approaches can provide important information about the crAsslike phages, but a true understanding of these highly abundant viruses (especially in terms of their biology, structure, and genomic features) will depend on our ability to grow them in the laboratory. Created using BioRender.com.

ECOLOGICAL MODELS

A number of different models have been described to explain bacteriophage lifestyles and their interactions with their hosts in their environment. While often used to describe phage-host dynamics in the ocean, they can be extrapolated to illustrate interactions within the human gut. The "piggyback the winner" model has traditionally been viewed as a switch from a lytic to temperate lifestyle, in order to keep host levels at a high abundance (*Knowles* et al., 2016). However, recent work has suggested that a healthy human virome is dominated by a lytic core of crAss-like phages and *Microviridae* (*Shkoporov* et al., 2019). It is possible that the use of mechanisms such as phase variation and delayed release of phage progeny by crAss-like phages correspond with alternative mechanisms of benign infection that align with the piggyback the winner model. These less aggressive means of replication may allow the bacterial population to persist to a high abundance, therefore enabling crAss-like phage persistence.

It is also possible that other models are at play, such as "Royal Family" dynamics. This is where the "kill the winner" model operates at the strain level, such that any fluctuations in phage or host populations would then go undetected (*Breitbart* et al., 2018). Recently, enrichment of nonsynonymous variants has been described in tail protein encoding genes of crAss-like phages (*Brown* et al., 2021; *Siranosian* et al., 2020). As tail proteins are often important mediators in bacterial host recognition, it is possible that crAss-like phages use these tail protein variants to switch hosts at the strain level, in line with Royal family dynamics. Possibly,

crAss-like phages use a number of different mechanisms from different models in combination, to enable them to persist stably, without affecting host abundance.

CONCLUSIONS

In just eight years, CrAss-like phages have gone from a viral unknown, to being recognised as the most abundant phages of the human gut, with sequences isolated all over the world. *In silico* analysis have revealed an array of interesting genomic features but many of their biological purposes remain unclear. Isolation of the first few phage-host pairs has allowed the elucidation of some of the biological characteristics of these phages (Figure 1). However, three of the five isolated crAss-like phages come from the Betacrassvirinae group, which have been shown to make up just < 1.5% of the crAss-like phages dominating the human gut (*Yutin* et al., 2021). CrAss-like phages from Alphacrassvirinae and Gammacrassvirinae groups dominate the gut virome, combined they make up just under half of all crAss-like phages. ΦCrAss002 is the only isolated crAssphage from the Alphacrassvirinae and it has shown to exhibit a number of biological mechanisms that are different from those of the Betacrassvirinae phages. It is likely that crAss-like phages employ a number of different mechanisms that allow them to persist so stably within the human gut. Isolation of members from other subfamilies will be required in order to garner further insights into these mechanisms. This will not be an easy task given that the hosts of these phages are Bacteroidetes, strict anaerobes that are difficult to isolate in their own right. The use of alternative genetic codes and the presence of introns and inteins in some of the crAss-like phage groups also add to the difficulty of correct annotation. It is vital that we continue to culture new members of this extensive and newly established phage order - the *Crassvirales*. Only by growing these phages in the laboratory will we begin to truly understand their role in the gut microbiota. An additional benefit will be structural studies that will allow us to assign functions to the many unannotated genes within crAssphage genomes. As the most abundant phages of the human gut, it is befitting that they should also become the most studied.

LITERATURE

Breitbart, M., Bonnain, C., Malki, K., and Sawaya, N.A.: Phage puppet masters of the marine microbial realm. Nat. Microbiol. 3, 754-766 (2018).

Brown, B.P., Chopera, D., Havyarimana, E., Wendoh, J., Jaumdally, S., Nyangahu, D.D., Gray, C.M., Martin, D.P., Varsani, A., and Jaspan, H.B.: crAssphage genomes identified in fecal samples of an adult and infants with evidence of positive genomic selective pressure within tail protein genes. Virus Res. 292, 198219 (2021).

Draper, L.A., Ryan, F.J., Smith, M.K., Jalanka, J., Mattila, E., Arkkila, P.A., Ross, R.P., Satokari, R., and Hill, C.: Long-term colonisation with donor bacteriophages following successful faecal microbial transplantation. Microbiome 6, 220 (2018).

Drobysheva, A.V., Panafidina, S.A., Kolesnik, M.V., Klimuk, E.I., Minakhin, L., Yakunina, M.V., Borukhov, S., Nilsson, E., Holmfeldt, K., Yutin, N., Makarova, K.S., Koonin, E.V., Severinov, K.V., Leiman, P.G., and Sokolova, M.L.: Structure and function of

virion RNA polymerase of a crAss-like phage. Nature 589, 306-309 (2020).

Dutilh, B.E., Cassman, N., McNair, K., Sanchez, S.E., Silva, G.G.Z., Boling, L., Barr, J.J., Speth, D.R., Seguritan, V., Aziz, R.K., Felts, B., Dinsdale, E.A., Mokili, J.L., and Edwards, R.A.: A highly abundant bacteriophage discovered in the unknown sequences of human faecal metagenomes. Nat. Commun. 5, 4498 (2014).

Dutilh, B.E., Schmieder, R., Nulton, J., Felts, B., Salamon, P., Edwards, R.A., and Mokili, J.L.: Reference-independent comparative metagenomics using cross-assembly: crAss. Bioinformatics 28, 3225-3231 (2012).

Edwards, R.A., Vega, A.A., Norman, H.M., Ohaeri, M., Levi, K., Dinsdale, E.A., Cinek, O., Aziz, R.K., McNair, K., Barr, J.J., Bibby, K., Brouns, S.J.J., Cazares, A., de Jonge, P.A., Desnues, C., Díaz Muñoz, S.L., Fineran, P.C., Kurilshikov, A., Lavigne, R., Mazankova, K., McCarthy, D.T., Nobrega, F.L., Reyes Muñoz, A., Tapia, G., Trefault, N., Tyakht, A.V., Vinuesa, P., Wagemans, J., Zhernakova, A., Aarestrup, F.M., Ahmadov, G., Alassaf, A., Anton, J., Asangba, A., Billings, E.K., Cantu, V.A., Carlton, J.M., Cazares, D., Cho, G.S., Condeff, T., Cortés, P., Cranfield, M., Cuevas, D.A., De la Iglesia, R., Decewicz, P., Doane, M.P., Dominy, N.J., Dziewit, L., Elwasila, B.M., Eren, A.M., Franz, C., Fu, J., Garcia-Aljaro, C., Ghedin, E., Gulino, K.M., Haggerty, J.M., Head, S.R., Hendriksen, R.S., Hill, C., Hyöty, H., Ilina, E.N., Irwin, M.T., Jeffries, T.C., Jofre, J., Junge, R.E., Kelley, S.T., Khan Mirzaei, M.K., Kowalewski, M., Kumaresan, D., Leigh, S.R., Lipson, D., Lisitsyna, E.S., Llagostera, M., Maritz, J.M., Marr, L.K., McCann, A., Molshanski-Mor, S., Monteiro, S., Moreira-Grez, B., Morris, M., Mugisha, L., Muniesa, M., Neve, H., Nguyen, N.P., Nigro, O.D., Nilsson, A.S., O'Connell, T., Odeh, R., Oliver, A., Piuri, M., Prussin li, A.J, Qimron, U., Quan, Z.X., Rainetova, P., Ramírez-Rojas, A., Raya, R., Reasor, K., O Rice, G.A., Rossi, A., Santos, R., Shimashita, J., Stachler, E.N., Stene, L.C., Strain, R., Stumpf, R., Torres, P.J., Twaddle, A., Ugochi Ibekwe, M.U., Villagra, N., Wandro, S., White, B., Whiteley, A., Whiteson, K.L., Wijmenga, C., Zambrano, M.M., Zschach, H., Dutilh, B.E.: Global phylogeography and ancient evolution of the widespread human gut virus crAssphage. Nat. Microbiol. 4, 1727-1736 (2019).

Guerin, E., Shkoporov, A.N., Stockdale, S.R., Comas, J.C., Khokhlova, E.V., Clooney, A.G., Daly, K.M., Draper, L.A., Stephens, N., Scholz, D., Ross, R.P., and Hill, C.: Isolation and characterisation of ΦcrAss002, a crAss-like phage from the human gut that infects Bacteroides xylanisolvens. Microbiome 9, 89 (2021).

Guerin, E., Shkoporov, A., Stockdale, S.R., Clooney, A.G., Ryan, F.J., Sutton, T.D.S., Draper, L.A., Gonzalez-Tortuero, E., Ross, R.P., and Hill, C.: Biology and Taxonomy of crAss-like Bacteriophages, the Most Abundant Virus in the Human Gut. Cell Host Microbe 24, 653-664 (2018)

Holmfeldt, K., Solonenko, N., Shah, M., Corrier, K., Riemann, L., VerBerkmoes, and N.C., Sullivan, M.B.; Twelve previously unknown phage genera are ubiquitous in global oceans. Proc. Natl. Acad. Sci. USA 110, 12798-12803 (2013).

Honap, T.P., Sankaranarayanan, K., Schnorr, S.L., Ozga, A.T., Warinner, C., and Lewis Jr, C.M.: Biogeographic study of human gut-associated crAssphage suggests impacts from industrialization and recent expansion. PLoS One 15, e0226930 (2020).

Hryckowian, A.J., Merrill, B.D., Porter, N.T., Van Treuren, W., Nelson, E.J., Garlena, R.A., Russell, D.A., Martens, E.C., Sonnenburg, J.L.: Bacteroides thetaiotaomicron-Infecting Bacteriophage Isolates Inform Sequence-Based Host Range Predictions. Cell Host Microbe 28, 371-379 (2020).

Knowles, B., Silveira, C.B., Bailey, B.A., Barott, K., Cantu, V.A., Cobián-Güemes, A.G., Coutinho, F.H., Dinsdale, E.A., Felts, B., Furby, K.A., George, E.E., Green, K.T., Gregoracci, G.B., Haas, A.F., Haggerty, J.M., Hester, E.R., Hisakawa, N., Kelly,

L.W., Lim, Y.W., Little, M., Luque, A., McDole-Somera, T., McNair, K., de Oliveira, L.S., Quistad, S.D., Robinett, N.L., Sala, E.S., Salamon, P., Sanchez, S., Sandin, S., Silva, G.G.Z., Smith, J., Sullivan, C., Thompson, C., Vermeij, M.J.A., Youle, M., Young, C., Zgliczynski, B., Brainard, R.A., Edwards, R., Nulton, J., Thompson, F., and Rohwer, F.: Lytic to temperate switching of viral communities. Nature 531, 466-470 (2016).

Liang, Y.Y., Zhang, W., Tong, Y.G., and Chen, S.P.: crAssphage is not associated with diarrhoea and has high genetic diversity. Epidemiol. Infect. 144, 3549-3553 (2016).

Manrique, P., Bolduc, B., Walk, S.T., van der Oost, J., de Vos, W.M., and Young, M.J.: Healthy human gut phageome. Proc. Natl. Acad. Sci. USA 113, 10400-10405 (2016).

McCann, A., Ryan, F.J., Stockdale, S.R., Dalmasso, M., Blake, T., Ryan, C.A., Stanton, C., Mills, S., Ross, P.R., and Hill, C.: Viromes of one year old infants reveal the impact of birth mode on microbiome diversity. PeerJ. 6, e4694 (2018).

Oude Munnink, B.B., Canuti, M., Deijs, M., de Vries, M., Jebbink, M.F., Rebers, S., Molenkamp, R., van Hemert, F.J., Chung, K., Cotten, M., Snijders, F., Sol, C.JA., and van der Hoek, L.: Unexplained diarrhoea in HIV-1 infected individuals. BMC Infect. Dis. 14, 22 (2014).

Porter, N.T., Hryckowian, A.J., Merrill, B.D., Fuentes, J.J., Gardner, J.O., Glowacki, R.W.P., Singh, S., Crawford, R.D., Snitkin, E.S., Sonnenburg, J.L., and Martens, E.C.: Phase-variable capsular polysaccharides and lipoproteins modify bacteriophage susceptibility in Bacteroides thetaiotaomicron. Nat. Microbiol. 5, 1170-1181 (2020).

Shkoporov, A.N., Clooney, A.G., Sutton, T.D.S., Ryan, F.J., Daly, K.M., Nolan, J.A., McDonnell, S.A., Khokhlova, E.V., Draper, L.A., Forde, A., Guerin, E., Velayudhan, V., Ross, R.P., and Hill, C.: The Human Gut Virome Is Highly Diverse, Stable, and Individual Specific. Cell Host Microbe 26, 527-541 (2019).

Shkoporov, A.N., Khokhlova, E.V., Fitzgerald, C.B., Stockdale, S.R., Draper, L.A., Ross, R.P., and Hill, C.: ΦCrAss001 represents the most abundant bacteriophage family in the human gut and infects Bacteroides intestinalis. Nat. Commun. 9, 4781 (2018).

Shkoporov, A.N., Khokhlova, E.V., Stephens, N., Hueston, C., Seymour, S., Hryckowian, A.J., Scholz, D., Ross, R.P, and Hill, C.: Long-term persistence of crAss-like phage crAss001 is associated with phase variation in Bacteroides intestinalis. BMC Biol. 19, 163 (2021).

Siranosian, B.A., Tamburini, F.B., Sherlock, G., and Bhatt, A.S.: Acquisition, transmission and strain diversity of human gut-colonizing crAss-like phages. Nat. Commun. 11, 280 (2020)

Walker, P.J., Siddell, S.G., Lefkowitz, E.J., Mushegian, A.R., Adriaenssens, E.M., Alfenas-Zerbini, P., Davison, A.J., Dempsey, D.M., Dutilh, B.E., García, M.L., Harrach, B., Harrison, R.L., Hendrickson, R.C., Junglen, S., Knowles, N.J., Krupovic, M., Kuhn, J.H., Lambert, A.J, Łobocka, M., Nibert, M.L., Oksanen, H.M., Orton, R.L., Robertson, D.L., Rubino, L., Sabanadzovic, S., Simmonds, P., Smith, D.B., Suzuki, N., Van Dooerslaer, K., Vandamme, A., Varsani, A. and Zerbini, F.M.: Changes to virus taxonomy and to the International Code of Virus Classification and Nomenclature ratified by the International Committee on Taxonomy of Viruses (2021). Arch. Virol. 166, 2633-2648 (2021).

Yutin, N., Benler, S., Shmakov, S.A., Wolf, Y.I., Tolstoy, I., Rayko, M., Antipov, D., Pevzner, P.A., and Koonin, E.V.: Analysis of metagenome-assembled viral genomes from the human gut reveals diverse putative CrAss-like phages with unique genomic features. Nat. Commun. 12, 1044 (2021).

Yutin, N., Makarova, K.S., Gussow, A.B., Krupovic, M., Segall, A., Edwards, R.A., and Koonin, E.V.: Discovery of an expansive bacteriophage family that includes the most abundant viruses from the human gut. Nat. Microbiol. 3, 38-46 (2018).

THE BIOLOGICAL EMPIRE OF THE BACTERIOPHAGE: A SUMMARY OF THE SEMINAR AND DISCUSSIONS AT THE 34TH OLD HERBORN UNIVERSITY SEMINAR

COLIN HILL

APC Microbiome Ireland, School of Microbiology, University College Cork, Cork T12 K8AF, Ireland

INTRODUCTION

The 34th Old Herborn University Seminar focussed on the biological empire of the bacteriophage (phage), bacterial viruses that represent the most abundant biological entities on Earth. Phage are not only the most abundant and most diverse entities in biology but they occupy an unusual position (or no position) on the tree of life. Given that they have a genetic basis (they can have ssRNA, dsRNA, ssDNA to dsDNA genomes of lengths varying from 2kb to over 1Mb) they are subject to evolution and are therefore very much part of the ongoing process of life on Earth, but because they are not metabolically active they cannot strictly be considered to be living. Notwithstanding their status as being alive or dead, they play a very important role in bacterial life and therefore in life on Earth. It has been estimated that up to half of all bacteria meet their end through an encounter with a phage. Phage can practice either a lytic or lysogenic lifecycle but in either event the end result is a lysed bacterial cell releasing many copies of the intact phage particle (there are exceptions to this, as is the case for almost any statement about phage, in that some bacteria can release filamentous phage while remaining intact).

The purpose of the 34th Old Herborn University Seminar was to gather a group of experts to examine some of the recent findings about phage and the role they may play in human health through their relationships with their target bacteria. Phage can be used in medicine in a manner similar to how we use antibiotics to target pathogenic bacteria causing infections, an approach usually termed 'phage therapy', but it is also likely that the 'virome' (the term used to describe the collection of phages and viruses resident in the microbiome) also plays a role in human health by influencing the composition and functionality of the bacteriome. On the first day of the Seminars the invited speakers presented recent data on various aspects of phage biology while day two consisted of an in-depth discussion of the presentations, involving both speakers and members of the scientific advisory board. In the following short account I will summarise some of the presentations and discussions but I encourage the reader to read the individual papers produced by each author for a more definitive and appropriately referenced account of their work.

PHAGE THERAPY

Volker Rusch opened the Seminar by welcoming the participants before our first speaker introduced the topic of phage therapy and the historic role of

the Eliava Institute. We had an ideal speaker in Mzia Kutateladze, the Director of the G. Eliava Institute of Bacteriophages, Microbiology and Virology, headquartered in Tbilisi, Georgia. Almost a century ago George Eliava worked at the Pasteur Institute in Paris with Felix d'Hérelle before returning to Georgia and setting up the Institute that bears his name. He was later joined by d'Hérelle in Tbilisi and together they learned how to exploit phage to treat infectious disease. The Eliava Institute has spent decades deploying phage to combat infection and have built a knowledge base unmatched elsewhere in the world. The strategy has been not only to develop personalised therapies but to create cocktails of phage that can be used across multiple patients. Currently the Eliava produces up to six of these cocktails for different indications, for example the Intesti phage preparation targets *Shigella*, *Salmonella*, enteropathogenic *E. coli*, *Proteus*, *Enterococcus*, *Staphylococcus* and *Pseudomonas aeruginosa*. Mzia spoke with passion about the excellent safety record with the use of phage as therapeutics but was clear as to some of the drawbacks in terms of a lack of appropriate placebos and the use of different preparations making it difficult to build a clear picture of their efficacy. She shared some impressive data from a number of field trials, some in soldiers on campaign and some conducted in large numbers of people in Ukraine and Russia in the 1970's, where phage treatment led to a significant reduction in the burden of illness. She finished by offering a hopeful vision of future trials in a number of countries on phage therapy.

The topic of phage therapy was continued with our two subsequent speakers, Martha Clokie from the University of Leicester and Graham Hatfull from the University of Pittsburgh. Martha focussed on one aspect of her work involving the clinical development of phage for treating urinary tract infections (UTIs). She highlighted the scale of the problem in that 400 million UTIs are diagnosed and 236,786 UTI-related deaths globally every year, and clearly established the unmet need for novel approaches. She and her team are approaching the issue with rigour and a clear strategy designed to bring phage into the clinic. She was also clear about the hurdles facing phage therapy approaches, from finding the right phage to proper formulation, delivery, efficacy and potential immunogenicity. She highlighted the ability of phage to work against biofilms, an important aspect of UTIs. Martha's group are keen to deploy ecological strategies for phage selection to get the best outcomes for patients. To this end they are utilising their knowledge of phage genomes to predict and select the most appropriate phage to use in cocktails. She also shared some exciting data derived from pig and chicken models to demonstrate the ability of phage to traverse the gastrointestinal tract and reduce pathogen counts. A really exciting presentation from a scientist at the forefront of this area of research.

Another leading scientist in the field is Graham Hatfull, a scientist whose work has generated headlines globally for one particular study involving a child with a lung transplant and an untreatable *Mycobacterium abscessus* infection. But first Graham outlined the SEA-PHAGES platform. This is an absolutely inspiring programme to encourage schoolchildren to find phage in their environments. Over 5,500 students take part each year and they even get to name the phage that they help to discover and characterise. But the highlight of Graham's talk was the story of how he and his group developed a phage cocktail, using both natural and engineered phage, to treat a disseminated

infection with antibiotic resistant *M. abscessus* in a 15-year-old girl. Graham outlined how a phage cocktail was personalized for *M. abscessus* GD01 and was subsequently delivered by intravenous administration at a dose of 10^9 PFU (plaque forming units), twice daily. He confirmed the personalised nature of the treatment in that the same cocktail does not work for most other strains, but encouragingly they saw no adverse reactions, and no phage resistance was observed. Graham was determined to emphasise that while the patient made a complete recovery and the infection was resolved, this remains an observation and does not remove the need for properly controlled clinical trials. Graham and his lab have gone on to produce phage for compassionate use for another 35 patients, but he stressed the need for properly controlled trials if phage therapy is to meet the needs of patients. He also told the audience that phage engineering may well be necessary to increase the range and efficacy of mycobacterial phage.

This concluded the 'phage therapy session' and the talks and the discussion on the following day all illustrated the promise of this approach for the treatment of infections, particularly antibiotic resistant infections. However, no one was underestimating the challenges that lie ahead for phage therapy to become mainstream in 'Western' medicine. The specificity of phage, their ability to multiply and therefore evolve within patients, the possibility of resistance development, issues with sceptical regulators were all topics that were discussed at length. But as might be expected from a group of scientists working with phage, there was more optimism than pessimism that phage therapy can be, if not a replacement, then at the very least an important adjunct to antibiotic therapy in the years to come.

PHAGE-HOST INTERACTIONS

Normally when we talk about phage and their hosts, we mean their bacterial hosts, but this session was dedicated to role of phage in impacting on the mammalian, mainly human, host. Three excellent speakers participated in this session, Paul Bollyky of Stanford University, Jeremy Barr from Monash University and José-Manuel Fernández-Real from the University of Girona. Paul got us started by explaining how the Pf phage that infects *Pseudomonas aeruginosa* can be considered to be a novel human pathogen. *P. aeruginosa* is a Gram-negative bacterium that causes skin and lung infections and is considered a critical priority pathogen by the World Health Organisation (WHO). The cost of treating *P. aeruginosa* infections has been estimated at >$1 billion/per annum. Paul explained that Pf phage is a lysogenic filamentous phage, an Inovirus with a ssDNA genome. Pf phage are one of those unusual phages that do not lyse their bacterial hosts but are extruded from the living cell. Paul went on to demonstrate that Pf phage contributes to *Pseudomonas* biofilm formation and bacterial colonization, and that intracellular Pf phage trigger innate immune responses that antagonize bacterial clearance. The Pf phage aggregate outside of the cell and confer a number of properties on the resultant biofilms that are informed, including making the cells more resistant to antibiotics. They also make sputum more adherent and viscous. The presence of phage can lead to tangling of the cilia on the surface of human

epithelial cells, thus impacting on the ability of cilia to clear an infection. The phages are present in a high percentage of *P. aeruginosa* infections in the lungs of patients with cystic fibrosis, and those patients suffer a more rapid decline in lung function over time. Paul was able to add weight to these observations in a series of elegant animal models of disease that showed that Pf phage are required to establish robust infection in mice. In part at least this could be ascribed to the ability of Pf phage to inhibit the production of tumour necrosis factor (TNF) in response to LPS. Paul's group was also able to show that Pf phage are internalised by mammalian cells and both associated with and contribute to the progression of wound infections. Even in the absence of their bacterial hosts Pf phage can inhibit wound healing.

Next up was Jeremy Barr, who set out to show us that phage can also enter mammalian cells, leading to transcriptional and immune responses to these internalised phage particles. Using cell line models Jeremy's group showed that phage can be found inside cells, in a cell-type dependent manner. The size of the phage particles also influenced uptake. Jeremy followed a detailed research strategy to investigate the means by which phage were trafficked in the cell, and the detailed response of the cell to this phage 'invasion'. The transcriptional profiles revealed a number of pathways that are much too complex to detail here, but really emphasised the sophisticated interactions between phage and the mammalian host.

Last in this session was José-Manuel, who gave a fascinating talk on the gut microbiome, bacteriophages and cognitive function. One of the most fascinating parts of a comprehensive presentation concerned a recent paper authored by José-Manuel and his group entitled 'Caudovirales bacteriophages are associated with improved executive function and memory in flies, mice and humans'. It comes as a surprise to most phage biologists that phage could play a role in executive function and memory in humans but José-Manuel made a compelling case that this is indeed true. Once again, I will leave it to his dedicated paper in this monograph to make the case more convincingly than I can on his behalf, but it is an exciting prospect for phage biologists to consider going forward.

PHAGE GENOMICS

The final session was delivered by myself and José Penades of Imperial College London. In my presentation I focussed on the challenge of turning the *in silico* phage genomes that are being reconstructed in sequencing projects into 'real' phage that can be studied in the lab. I gave the example of the crAssphage, the most abundant phage in the human gut but which until recently had never been grown in the lab. Scientists in my group, led by the talented Andrey Shkoporov, managed to identify *Bacteroides intestinalis* as the host for crAss001. This phage is approximately 100kb in size and practices an unusual lifecycle in that it co-exists with its target host in culture, both in the lab and in animal models. This mimics what we see in human longitudinal studies where the phage and its host co-exist over 12 months in different individuals. The underlying mechanism involves switching between different capsular polysaccharides that act as a receptor for the phage, ensuring that a mix of both sensitive and resistant hosts are present at all times. This almost certainly impacts the

functionality of the host in the gut since these surface structures are also the interface between the bacterium and its neighbours, and its mammalian host. Being able to grow the phage also permitted the structural analysis of the phage using cryo-EM, which also revealed several novel features in this podovirus. The benefits of turning *in silico* phage into 'real' phage will have to be realised for many additional phage genomes in years to come, a significant challenge for phage scientists.

José Penades gave a fascinating talk on how phage can mobilise pathogenicity islands encoding immune systems as weapons to eradicate competitors. José works with *Staphylococcus aureus*, a talented pathogen that encodes a number of pathogenicity islands (SaPIs). Many of these virulence genes are located on phage inducible chromosomal islands (PICIs) that can be mobilised at high frequency by phage. While these were discovered in *S. aureus*, they are now believed to be widespread among across the bacterial Kingdom. José showed us some elegant work that demonstrated that some PICIs can encode phage resistance mechanisms that can even block prophage induction and horizontal gene transfer. This is an underappreciated aspect of phage biology and it was truly exciting to get a glimpse of this hidden world where bacterial competition is supported by phages.

DISCUSSION

It was a real pleasure to participate in the discussion session that was both lively and informative. It is rare that scientists get the chance to go back over a session with the speakers, exploring the synergies and points of difference in the previous days' seminars. We took full advantage of it, dissecting the finer points of the talks, and enjoying the debates that ensued. The objective was not to reach consensus but all were agreed that phage therapy offers an important tool for our clinical colleagues, but one that is faced with significant challenges to deliver on that promise. The role of phage in interacting with the mammalian host was also highlighted as an under-researched aspect of phage biology. This is an area that would have been regarded with scepticism until recently but is now becoming an accepted aspect of how phages can potentially impact on the health and even executive function of the mammalian host. Lastly, we were all agreed (when would scientists not agree on this point?) that more fundamental research in needed and that we are only scratching the surface of the extraordinary diversity and importance of the role of phage in shaping and influencing bacterial communities, and therefore human health.