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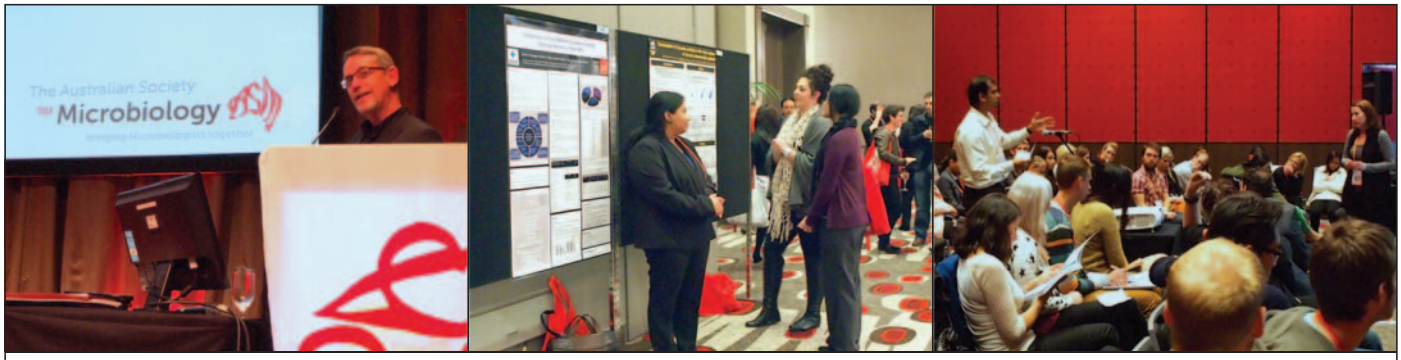
Bacteriophages



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Contents

Cover image: Cryo-electron tomography of Staphylococcus aureus *cell infected by a bacteriophage (see Bárdy* et al. *in this issue) and phage therapy products from the Eliava Institute.*

Dena Lyras
President of ASM

Since this is my first communication with you this year I will start by wishing you all a happy new year and extend my best wishes to all of you for 2019.

The ASM Council and Executive Committee had a very productive meeting in February to review our activities and to plan ahead for the coming year, and I would like to bring to your attention several new and ongoing initiatives that give our members maximum value for their membership.

I am excited to announce the new ASM standing committee for Professional Development that will be chaired by A/Prof Priscilla Johanesen, the current student and early career microbiologist engagement co-ordinator. The Standing Committee is currently being formed and will consist of eight additional members including the Chair of the Workforce Standing Committee, the Chair of the Standing Committee on Clinical Microbiology, and the Convenor of the Education SIG. The role of the committee is to foster the development of student and early career microbiologists of the Society, including researchers and working microbiologists. This initiative will involve engaging students and early career microbiologists in a way that improves their careers by providing resources, activities and opportunities that will bring these members together and help to foster their professional growth and development. In this way we hope to support these members and enhance their overall experience of ASM.

Note also that a new initiative for 2019 includes an annual teacher's travel award, valued at $4000, to attend the American Society for Microbiology Conference for Undergraduate Educators (ASM-CUE), which is the world's premier microbiology teachers' conference. As we did for the first time in 2018, we are also providing 100 travel awards, each valued at $200, to make it easier for members within 10 years of attaining their highest qualification to attend our Annual Scientific Meeting. Our other awards are also on offer and have a closing date of 31 March, so do take the opportunity to apply or to encourage those around you to apply, or nominate others.

As we begin to approach the middle of the year, I am very much looking forward to seeing all of you at our national meeting in Adelaide (http://asmmeeting.theasm.org.au/), which will be held from 30 June to 3 July. We are in the final planning stages for the scientific and social program and the local organising committee and ASM executive committee are working hard to bring you an excellent meeting in every aspect. I can also announce that our next national meeting will be held in Melbourne and that planning is already well underway for 2020.

I would also like to congratulate and thank Dr Deirdre Gleeson and her committee for organising a very successful AusMe (Australian Microbial Ecology) conference, which was held over 11–13 February in 2019 in Western Australia. AusMe is an ASM initiative and the first meeting in 2017 was a wonderful success, with about 100 attendees. The meeting this year drew over 140 attendees and was very well received by all. The next AusMe will be held in 2021 with details to come; it is the perfect meeting to attend if you have an interest in any aspect of microbial ecology. Note also that one of our partner meetings, BacPath, is also being held this year from 30 September to 3 October in Western Australia. BacPath is a wonderful meeting for those interested in bacterial pathogenesis and molecular bacteriology, I encourage you to visit their website for more details (http://www.bacpath.org/).

Finally, I would personally like to thank members of ASM Council, the Executive Committee and state branch committees for the significant time and effort they invest in organising all of the activities described above. All of these people give their personal time to these activities and I would like to recognise them for making the Society the success that it is. Thank you.

As always, please visit our website www.theasm.org.au to access information regarding upcoming meetings and awards. You may also like to follow, and contribute to ASM on Twitter, @AUSSOCMIC, or on Facebook to make sure you keep up with the latest news, trends and developments in Microbiology in Australia and around the world.

Bacteriophages



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In order to avoid a return to the pre-antibiotic era, alternative treatments to combat microbial diseases are urgently needed. In this context, bacteriophages, which have been used effectively in distant parts of the world during the cold war era, are now gaining significant interest in the West. This special issue of the *Microbiology Australia* thus focusses on bacteriophages with contributions from Australia and from the members of the *Expert round table on acceptance and re-implementation of bacteriophage therapy.*

During the International Conference titled 'Bacteriophages as tools for therapy, prophylaxis and diagnostics', which was held on 19–21 October 2015 in Tbilisi, Georgia at the Eliava Institute[1], an 'Expert Round Table' was held by multidisciplinary scientists discussing the acceptance and re-implementation of bacteriophage therapy. Since 2015, the 'Expert Round Table' participants published opinion papers[2,3] including one of the most downloaded *Microbiology Australia* articles on the '*Application of Bacteriophages*'[4].

Expert round table on acceptance and re-implementation of bacteriophage therapy members at the Eliava Institute, Tbilisi, Georgia in October 2015.

Within Australia, a Bacteriophage Biology & Therapeutics Special Interest Group (SIG) was recently formed under the umbrella of Australian Society for Microbiology. This issue is thus put together by international participants from the 'Expert Round Table', contributions from Bacteriophage Biology & Therapeutics SIG and members of the ASM.

The issue includes an article from Rustam Aminov and Nina Chanishvili outlining the antibiotic resistance problem and the urgent need for alternative therapies. Jean-Paul Pirnay, Daniel de Vos and Gilbert Verbeken give an overview on the clinical applications of bacteriophages in Europe. Keith Potent and Carola Venturini, Alexsandra Petrovic Fabjian and Ruby Lin complement two articles describing bacteriophage therapy and its processes within Australia. Shawna McCallin, Jessica Sacher and Jen Zheng touch on an important aspect of bacteriophage therapy by providing examples on their 'compassionate use'. Wai Hoe Chin and Jeremy Barr take us into the depths of phage biology with their article titled 'Phage biology in 'organ-on-chip' devices'. Martina Jones tells us about the 'use of bacteriophage for antibody discovery'. Son Tuan Le and Ipek Kurtböke touch the environmental applications of bacteriophages in their article related to the use of bacteriophages as biocontrol agents in aquaculture settings. Finally, an advanced understanding on the biology of bacteriophages comes from Pavol Bárdy, Dominik Hrebik, Roman Pantućek and Pavel Plevka on the 'Future prospects of structural studies to advance our understanding on phage biology'. In a Lab Report, University of the Sunshine Coast students Rhianna O'Regan and Annaleise Wilson present their work on the biological control of *E. coli* contaminating herbs using bacteriophages.

Jeremy Barr also presents an introduction to the recently established Bacteriophage Biology & Therapeutics SIG Group, which interested Australian researchers are encouraged to join.

The inset photo on the cover for the issue is contributed by the Eliava Institute where bacteriophages are available in their pharmacy for public use.

We conclude by conveying the kind greetings of the Bacteriophage Biology & Therapeutics SIG and the 'Expert Round Table' participants from overseas.

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Bacteriophage therapy: coping with the growing antibiotic resistance problem

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The global problem of multidrug-resistant bacterial pathogens requires urgent actions, including the development of therapies supplementary or alternative to antibiotics. One of the infection control options could be phage therapy. This article gives a brief overview of phage therapy potentials as well as the challenges it faces in order to become a widely accepted form of infection treatment.

The history of antimicrobial drug discovery includes more than 15 classes of antimicrobials that became a cornerstone in microbial infection control and management and saved many lives[1]. Antimicrobial therapy indeed became one of the most successful forms of therapy in clinical medicine. However, the broad and often indiscriminate use of antimicrobials in human and veterinary medicine and in agriculture resulted in the widespread antimicrobial resistance in microbiota of many ecological compartments[2]. Especially worrisome is the rise of multidrug resistance among bacterial pathogens, which may severely limit our abilities to control infectious diseases. Very limited options, for example, exist to treat the so-called ESKAPE bacteria (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species)[3]. If no immediate actions are taken, the estimated death toll due to multidrug-resistant bacterial pathogens may reach 10 million by the year 2050[4].

The question is what went wrong with this initially very successful form of infectious disease treatment? Why resistance to antibiotics develops so rapidly? To understand this phenomenon, we have to take a closer look into the fundamental biological processes governing the ecology and evolution in microbial ecosystems. For a long time, the problem of antimicrobial resistance has been viewed in isolation, mainly from the clinical microbiology perspective, e.g. as associated exclusively with the use/overuse/misuse of antimicrobials in human medicine. Admittedly, this can be one of the factors contributing to the dissemination of antimicrobial resistance, but the problem has much wider implications and must be contemplated within a broader evolutionary and ecological context.

In natural ecosystems, antibiotics play an essential role in regulatory processes that are involved in many functions of microbial ecosystems[5]. While serving as signalling molecules at low concentrations in natural ecosystems[6], in human and animal infectious disease therapy they are mainly used for their bacteriolytic and bacteriostatic activities expressed at high concentrations. Also, they are widely used at subtherapeutic concentrations in food animal production for metaphylactic purposes. Extensive use of antibiotics in clinical medicine, veterinary, agriculture and other applications are the hot spots with persistent antibiotic influx into microbial ecosystems. These are the places, where naturally occurring antibiotic resistance genes are selected and amplified. At this stage, antibiotic resistance genes are integrated into the normal microbiota and the fitness cost associated with the antibiotic resistance gene carriage is reduced. Because of this, antibiotic resistance becomes very resilient against eradication, even in the absence of antibiotic selective pressure[7]. The pool of the antibiotic resistance genes, which is amplified at these hot spots is then released, together with the concomitant antibiotics, into other ecological compartments. These are further disseminated to even more distant ecological compartments, including pathogens, via extensive horizontal gene transfer (HGT) mechanisms[8].

The widespread antibiotic resistance in a variety of microbiota, including human and animal pathogens, is the consequence of extensive use of antibiotics in human and veterinary medicine and agriculture. What can be done to limit and contain it? It is clear that the massive antimicrobial usage, which inevitably results in selection of the corresponding resistance mechanisms, has to be lessened. Unfortunately, the trends in antibiotic production and consumption are quite opposite, suggesting a very substantial growth for both humans and animals[9,10].

All major classes of antimicrobials were discovered during the golden age of antibiotic discovery, which came to an end more than 50 years ago[1]. Since then, the main antimicrobial drug developments included extensive modifications of the existing natural compounds, which, however, cannot guarantee the rapid development of resistance even against the newer antimicrobial derivatives. There are many potential avenues for the development in addition to modification of the existing antimicrobials. Alternatives to antimicrobials are urgently needed, and one of the most promising approaches could be the phage employment.

The idea to treat infections with phage came out of the pioneering work of Félix d'Hérelle[11]. The discovery of antibiotics that offered more convenient means to control infectious diseases, however, overshadowed the phage therapy approach. It has been largely abandoned except in a several countries: Georgia, Poland, and Russia, where it has remained as a part of authorised therapy for treatment of certain bacterial infections. A renewed interest in phage therapy is dictated by the need of new approaches to control bacterial infections, especially multidrug-resistant, and by its advantages.

First, unlike the wide range of bacteria targeted by antibiotics, phages are very specific and do not affect other beneficial microbes. This prevents complications such as antibiotic-induced dysbiosis and secondary infections. Second, phages multiply at the sites where the targets are present thus amplifying the local antibacterial effects. Third, no side effects of phage therapy have been so far detected[12–14]. Fourth, phage-resistant bacteria remain sensitive to other phages and, according to post-soviet regulations and standards for production of commercial phage preparations, introduction of new phages is a much faster and cheaper process compared to the development of new antimicrobials. Fifth, phages may be a valuable source of enzymes, such as lysins, active against pathogens[15]. Sixth, bacteriophages could play a significant role in restricting the evolution and spread of antibiotic resistance[16,17]. Seventh, phages may be effectively used for diagnostic purposes[18]. Eighth, unlike antibiotics, phages are efficient against biofilm-forming pathogens. And finally, antibiotics can be complemented by phages: phage-antibiotic combined therapy of infections is more effective than either alone[19]. In practical terms, the recent approval of bacteriophages as food additives to control foodborne pathogens opened new opportunities for their use in 'biocontrol' processes[20].

Although phages have been applied for treatment of various diseases for a century, as mentioned above their use in practical medicine is still limited to several countries. Safety is one of the main concerns when considering the use of phages for therapy and prophylaxis since, unlike regular pharmacological products, they are living organisms. Besides, they may contribute to HGT in the form of transduction[21]. According to the technological requirements applicable in the countries currently manufacturing commercial phage preparations, before a new phage is considered for practical use, it must pass a number of tests confirming its lytic nature and ruling out a potential involvement in HGT. The advance of omics technologies helps to address this issue. Genome sequencing is an essential initial step for considering phage candidates since it identifies prophage genes such as integrases, repressors, excisases, recombinases, terminases and hence, allows predictions of potential prophage properties including virulence factors or prophage incompleteness[22].

Regulatory issues represent a major obstacle for the implementation of phage therapy: its efficacy has to be confirmed according to the current pharmacological standards. This requires that properly designed, randomised, placebo controlled, and double-blind clinical trials have to be performed. So far, 17 clinical trials have been registered between 1996 through 2018, but the majority of them could not recruit enough patients; the others were not well designed, therefore they fall far from providing statistically relevant conclusions about the efficacy of phage therapy[22]. For instance, the recently completed Phagoburn trial, which represented a public investment of 3.85 million euros, enrolled a total of 27 patients only among 11 centres[23]. This is much less than the pre-calculated 220 patients needed to provide statistically significant results for the study. Besides, the trial was designed for treatment of burn wound infections caused by *E. coli*, but the clinical results showed that *P. aeruginosa* was predominant. The inadequate trial outcome was significantly affected by the initial error in trial design as it is well known that burn wounds are mostly infected by *P. aeruginosa*, not *E. coli*. A randomised, placebo-controlled, double-blind clinical trial has been recently completed in Georgia. Patients with urinary tract infections caused by *E. coli*, *Enterococcus* spp., *Proteus* spp., *Streptococcus* spp., *S. aureus* and P. *aeruginosa* were enrolled in the study. The patients were treated with a commercial preparation

Pyo-bacteriophage, which is a mix of bacteriophages targeting all these pathogens. Preliminary results of the clinical trial have been published[24,25], which hopefully will result in a broader recognition of phage therapy for treatment of multidrug resistant infections. Once widely accepted, phage can be used as a valuable alternative option to lessen antibiotic usage and corresponding resistance.

Conflicts of interest

The authors declare no conflicts of interest.

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Biographies

Prof Nina Chanishvili has vast experience working in the field of bacteriophage research and phage therapy. Her recent projects were dedicated to clinical studies of bacteriophage application for treatment of urinary tract infection; selection of bacteriophages against MDR Salmonella spp.; study of the potential role of *B. fragilis* bacteriophages for prophylaxis of colon cancer. She is full professor at the New Vision University (Tbilisi, Georgia) and an invited lecturer at the Iv. Javakhishvili Tbilisi State University. Prof Nina Chanishvili is a peer reviewer of the scientific journals: *Frontiers in Microbiology, Viruses, Pharmaceuticals*, *Antibiotics*, etc. She is a Vice President of the Georgian Association for General and Applied Microbiology (GAGAM) and a Board Member of the Eliava Foundation-Association for Development of Bacteriophages in Georgia. In 2000 she has been awarded by the American Society of Microbiology with the Morrison-Rogoza Award. She is an author of over 120 research articles, book chapters, abstracts and monographs.

Dr Rustam Aminov's main research interests are in antimicrobials, antimicrobial resistance and in gut microbiology. He has published more than 120 articles and book chapters and edited several research topics on these subjects. He serves as editor of *Frontiers in Microbiology* and *FEMS Microbiology Letters*. He is currently affiliated with the University of Aberdeen and Kazan Federal University, and is the recipient of several fellowships from JSPS.

Clinical application of bacteriophages in Europe



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Bacteriophages could help address the antibiotic resistance crisis that impacts health systems all over the world. In 2011, the European Commission formally confirmed that phage products used as therapeutics are medicinal products and thus manufacturers need to navigate the extremely arduous and enormously expensive medicine development and marketing pathway. However, up until now, not one therapeutic phage product has made it to the European market, and yet clinicians are under increasing pressure to use phages in the treatment of multidrug-resistant bacterial infections. While a handful of small European enterprises are struggling to squeeze therapeutic phage products through the conventional and centralised European medicinal products funnel, some clinicians and academics are exploring (European) national solutions to accelerate the availability of phages for the treatment of an increasing number of desperate patients. This mini-review summarises the actual status and perspectives of clinical phage application in Europe.

Two decades before the advent of antibiotics, bacteriophages (or phages) were sporadically used to treat bacterial infection across the world. By 1940, therapeutic phage preparations were commercialised by renowned pharmaceutical companies such as Eli Lilly. After World War II, broad spectrum antibiotics were established as the antibacterial agents of choice, but isolated from Western advances in antibiotic production, scientists in the Soviet Union continued to develop phage therapy, with the George Eliava Institute in Tbilisi, Georgia, as the epicentre of these activities. Global pandemics of antibiotic resistant bacteria, causing the death of hundreds of thousands of patients, demonstrate the need for a sea change in antibacterial treatment policy. Today, in the Western world, phage therapy is brought out of mothballs to come to the aid of patients being increasingly failed by antibiotics. The renewed interest in phage therapy research is illustrated by an exponential increase in phage therapy-related papers in medical literature (Figure 1). Unfortunately, the reintroduction of phage therapy in Western medicine is not running smoothly[1–3]. In this paper we summarise the state of affairs in Europe.

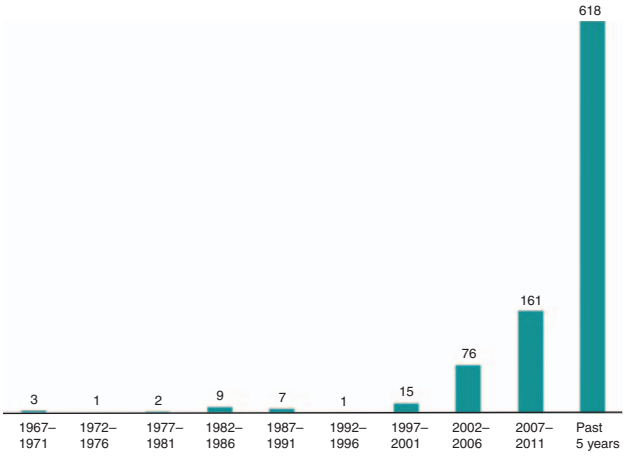

Figure 1. PubMed search results for the terms 'phage therapy' or 'bacteriophage therapy' across time periods.

Phages are medicinal products

In the European Union (EU), the rediscovered phage preparations were classified as 'Medicinal Products' – 'Drugs' in the United States (US)[4]. In February 2011, two Members of the European Parliament, Ivo Belet (Belgium/PPE) and Catherine Trautmann (France/S&D) raised the question to the European Commission and Council of how bacteriophage therapy should be regulated in Europe and whether the Commission would consider creating an extra 'Bacteriophage Therapy' section in the EU Medicinal Product framework[4]. On 29 March 2011, the European Commissioner for Health and Consumer Policy, Mr Dalli, answered on behalf of the European Commission: 'The EU's legislation on medicinal products does not define specific requirements related to bacteriophage therapy or medicines composed of bacteriophages.' He added, 'the Commission considers that the existing regulatory framework is adequate for bacteriophage therapy without the need for an extra set of documentation'. The EU phage classification was based on a blinkered application of the Medicinal Product definition:

a) any substance or combination of substances presented as having properties for treating or preventing disease in human beings; or
b) any substance or combination of substances which may be used in or administered to human beings either with a view to restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic action, or to making a medical diagnosis.

The EU legal framework for medicinal products was primarily laid down in 2001 in Directive 2001/83/EC and Regulation (EC) No 726/2004, and was developed to guarantee high standards of quality and safety, but also to promote the EU internal market with measures that encourage innovation and competitiveness in Europe. A large body of Medicinal Product requirements were implemented and progressively harmonised across the whole European Economic Area, roughly and generally (there are exceptions) consisting of:

- Manufacturing according to good manufacturing procedures (GMP)
- Preclinical studies
- Phase I, II and III clinical trials
- Centralised marketing authorisation (granted by the European Medicines Agency or for certain types of medicines by the National competent authorities)

As a result, the large body of costly and time-consuming requirements and procedures for manufacturing and for obtaining marketing authorisation for conventional medicinal products for human use were also imposed on phage therapy medicinal products (PTMPs). These requirements were developed to cater for widely used and industrially produced static (immutable) drugs such as aspirin and antibiotics, but are less suitable for sustainable, customised, phage therapy approaches[5,6]. Technically speaking, pre-defined PTMPs, produced on an industrial scale, could make it through the medicinal product funnel – minding some adaptations, but it is unlikely that such preparations will be able to timely deal with changes in the incidences of infecting bacterial species in certain settings or geographical areas and with the inevitable emergence of phage-resistant clones[7]. The efficacy of PTMPs is therefore likely to decrease over time, requiring regular adaptions and re-approval (of the new PTMP) for extended use. Multiple phage types are usually needed to treat the different clinically relevant strains of one bacterial species. Furthermore, several bacterial strains are often present in an infection. Therefore, to acquire a more or less broad level of activity, phage cocktails harbouring many different phages will be required. Ideally, therapeutic phages need to be tested for effectiveness against the patients' pathogens (a 'phagogram') and individually prepared. Intermediate or combined (industrially prepared and personalised phage preparations) approaches might be feasible[5]. Unfortunately, it turns out that the established pharmaceutical industry is not interested in PTMPs, mainly because of limitations in intellectual property protection of a technique that is in the public domain since the 1920s and uses 'products of nature' such as phages, and because of the above-mentioned phage specificity and bacterial resistance issues, which compromise widespread and long-term use of immutable pre-defined PTMPs. In the absence of government initiatives, it is left to a handful of small and medium enterprises (SMEs) to develop these PTMPs, using venture capital and/or public funding[2].

Randomised controlled trials

The European Medicines Agency (EMA), based in Amsterdam, underpins the centralised authorisation procedure. The Agency also guarantees a constant exchange and flow of information regarding the scientific assessment of medicinal products in the EU. It is in this context that the EMA organised a workshop on the therapeutic use of bacteriophages in London, on 8 June 2015. At the end of the workshop, EMA emphasised that a medicine cannot be recommended for approval before its efficacy and safety have been proven on the basis of appropriately designed clinical trials[8]. Several formal clinical trials were launched in Europe (Table 1), but none of them managed to avowedly prove a sufficient efficacy of PTMPs. Note that, by default (EU classification), most of these clinical trials evaluated PTMPs using conventional clinical trial designs, but did NOT evaluate long-established flexible and often personalised phage therapy approaches using regularly updated phage preparations. Different phase I, I/II and II clinical trials did however demonstrate the safety of PTMPs, which is

Table 1. Controlled clinical trials involving the application of phages to humans in Europe.

Study title	Conditions	Interventions	Locations (countries)	Status
A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant *Pseudomonas aeruginosa*[8]	Chronic otitis	Anti-*P. aeruginosa* bacteriophage preparation	United Kingdom	Completed
Standard treatment associated with phage therapy versus placebo for diabetic foot ulcers infected by *Staphylococcus aureus*[A]	Diabetic foot – staphylococcal infections	Topical anti-*Staphylococcus* bacteriophage therapy	France	Not yet recruiting
Experimental phage therapy of bacterial infections[A]	Bacterial infections	Bacteriophage preparation	Poland	Unknown
Evaluation of phage therapy for the treatment of *Escherichia coli* and *Pseudomonas aeruginosa* wound infections in burned patients[A,B]	Wound infection	*E. coli* and *P. aeruginosa* phage cocktail Control: standard of care: silver sulfadiazine	Belgium, France, Switzerland	Completed
Bacteriophage effects on *Pseudomonas aeruginosa*[A]	Cystic fibrosis	Collection of induced sputum in order to evaluate the efficacy of a cocktail of 10 bacteriophages	France	Completed
Existence in the human digestive flora of phages able to prevent the acquisition of multiresistant *Enterobacteria*[A]	Multiresistant *Enterobacteriaceae*	Stool collection and screening for multi-resistant bacteria and bacteriophages directed against the strains of the patient bearing	France	Not yet recruiting
Phages dynamics and influences during human gut microbiome establishment[A]	Human gut microbiome development	Collection of diapers with fresh stools	France	Recruiting
Probiotics after discharge[A]	Microbiota – bacteriophages – infantile colic – growth	Dietary supplement: probiotic	United Kingdom	Recruiting
The role of phages in microbial gut ecology: a study of interactions between antibiotics and the gut microbiota[B]	Healthy volunteers	Parallel, single-blind, randomised, controlled study	Denmark	Completed
Chronic ulcers (TP-102)[C]	Chronic ulcers	Phase I clinical trial Phage cocktail to treat diabetic wound infections	Portugal	Initiated

[A]Clinicaltrials.gov (https://clinicaltrials.gov/).
[B]Clinicaltrialregister.eu (https://www.clinicaltrialsregister.eu/ctr-search/search).
[C]TechnoPhage.pt (http://www.technophage.pt/index.php/r-d/product-pipeline).

consistent with the safety data provided by numerous preclinical animal studies. To date, two European randomised controlled phase I/II clinical trials showing some phage treatment efficacy have been reported in literature. In the first one, phage therapy against chronic *Pseudomonas aeruginosa* otitis was investigated. The bacterial load was significantly lower in 12 phage-treated patients as compared to the 12 placebo-treated patients and no adverse effects were observed[9]. The second one is the clinical phase II Phagoburn trial (http://www.phagoburn.eu), designed to evaluate the treatment of *P. aeruginosa* and *Escherichia coli* infected burn wounds using two dedicated phage cocktails[10]. Cocktails of no less than 12 and 13 phages were needed to ensure a certain

activity against pre-defined collections of *P. aeruginosa* and *E. coli* isolates, respectively. Manufacturing of one batch (according to GMP) of the investigational PTMPs took 20 months and the largest part of the study budget and only a very small number of phages (10–100 PFU/mL instead of the anticipated 10^6 PFU/mL) was actually applied due to stability problems of the phage cocktails. In addition, phage specificity issues hampered the recruitment of patients. Because each of the two study products, which couldn't be applied simultaneously, targeted only one of the multiple bacterial species that are known to (simultaneously) infect or colonise burn wounds, physicians were reluctant to include patients[11]. Only 27 patients were enrolled in the *P. aeruginosa* arm of the study and the *E. coli* arm was stopped (only one patient was enrolled). At very low concentrations, the *P. aeruginosa* phage cocktail was shown to decrease the bacterial load in burn wounds, but at a slower pace than the standard of care (silver sulfadiazine cream). Further studies using higher phage concentrations and selected phage preparations, taking into account the results of 'phagograms', in a larger sample of participants are warranted. Regardless of the final clinical outcome of the PhagoBurn study, it showed that dedicated and realistic production and documentation requirements and treatment protocols are urgently needed.

Article 37 of the Declaration of Helsinki

The bottom line is that today there are no PTMPs on the EU market and that the pressure on clinicians to apply phage therapy in desperate cases is increasing, fuelled by an increasing promotion of phage therapy in the media (e.g. eulogising documentaries in prime time). However, even when confronted to serious public health threats, such as the 2011 *E. coli* O104:H4 outbreak in Germany[12], competent authorities are reluctant to authorise the use of non-licensed phage therapy preparations. During this lethal foodborne epidemic, which took the life of 54 patients, Nestlé Research Centre offered a lytic phage to the German public health sector, but this phage was ultimately not used[13]. Awaiting commercially available licensed PTMPs, some European patients suffering from chronic, extremely resistant or difficult to treat bacterial infections are travelling to phage therapy centres abroad, such as the Eliava Phage Therapy Center in Tbilisi, Georgia.

In addition, sporadic 'non-PTMP' phage applications were carried out in Europe, often under the umbrella of Article 37 (Unproven Interventions in Clinical Practice) of the Declaration of Helsinki (www.wma.net)[14,15]:

> *In the treatment of a patient, where proven prophylactic, diagnostic and therapeutic methods do not exist or have been ineffective, the physician, with informed consent from the patient, must be free to use unproven or new prophylactic, diagnostic and therapeutic measures, if in the physician's judgment it offers hope of saving life, re-establishing health or alleviating suffering. This intervention should subsequently be made the object of research, designed to evaluate its safety and efficacy. In all cases, new information must be recorded and, where appropriate, made publically available.*

Even though no safety issues were reported and most targeted infections seemed to have been resolved, the low number and diversity of these 'Helsinki' phage therapy cases does not allow one to unambiguously demonstrate that the positive clinical outcome was related to the use of phages. For instance, with the exception of a reported use of phages in the treatment of *P. aeruginosa* septicaemia in a patient with acute kidney injury[15], most patients were given other potent anti-bacterials together with the phage preparations.

Poland: experimental treatment

In Poland, a member of the EU, phage therapy is considered an 'Experimental Treatment', covered by the adapted Act of 5 December 1996 on the Medical Profession (Polish Law Gazette, 2011, No. 277 item 1634) and Article 37 of the Declaration of Helsinki[16,17].

Experimental phage treatments are possible in Poland minding the following requirements:

- Written informed consent of the patient (or legal representative)
- Approval by an institutional review board (bioethics commission)
- Phages can only be applied by a qualified doctor
- Phages can only be applied when other available treatments have failed

In June 2005, the Ethical Committee of the Medical Academy in Wroclaw authorised a study named 'Experimental Phage Therapy in Bacterial Infections' (Table 1). Neither the EU Medicinal Product Regulation nor its Polish National translation was applied and the EU did not oppose. As such, the Phage Therapy Unit of the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy in Wrocław (an institute of the Polish Academy of Sciences) offers phage therapy to treat patients infected with drug-resistant bacteria. In 2012, Międzybrodzki *et al.*[18] presented a detailed retrospective analysis of the results of phage therapy of 153 patients with a wide range of infections, resistant to antibiotic therapy, admitted for treatment at the Wrocław Phage Therapy Unit between January 2008 and December 2010. They suggested that phage therapy had provided good clinical results in a significant cohort of patients with otherwise untreatable chronic bacterial infections.

France: compassionate use

Compassionate use is a treatment option that allows the use of an unauthorised medicine. Under strict conditions, products in

development can be made available to groups of patients who have a disease with no satisfactory authorised therapies and who cannot enter clinical trials (https://www.ema.europa.eu/en/human-regulatory/research-development/compassionate-use). The EMA provides recommendations, but the practice of 'compassionate use' is actually coordinated and implemented by the Member States, and there is some variation in national rules and procedures. Much like article 37 of the Declaration of Helsinki, the compassionate use treatment option or programmes can only be put in place if the medicine, the phage *in-casu*, is expected to be of help in life-threatening, long-lasting (chronic) and/or seriously debilitating illnesses that are not treatable using the current armamentarium. In principle, the compassionate approach can only apply to medicinal products that are being tested or have entered the marketing authorisation application process after early study results have shown efficacy and safety, but have not yet been approved. In France, phages have been used under the umbrella of the compassionate use setting. A recent publication describes a number of cases of patients treated compassionately with phages with a focus on osteoarticular infections during the past 10 years[19]. In practice, a multidisciplinary team (surgeons, infectiologists, microbiologists and pharmacists) discussed the potential compassionate phage application and compiled a medical dossier and specific treatment protocol, in consultation with the patient (or his legal representative) and the treating hospital's ethical committee. However, since 2016 the 'Agence Nationale de Sécurité du Medicament et les Produits de Santé (ANSM),' the French competent authority, is also tightly involved. ANSM created a specific committee 'comité scientifique spécialisé temporaire (CSST)' for phage therapy, which is composed of (external) experts in different fields. Their task is to specifically evaluate and guide the phage therapy requests sent to the ANSM. They meet on a regular basis and will remain active as long as the problem exists. The requests for phage applications are discussed in dialogue with the treating physicians and a consensus advice is transmitted to the ANSM, who will or will not authorise the request. From 2006 to 2018, 15 patients were treated compassionately with phages in France. Eleven were immediately cured[19]. These compassionate phage treatments, under supervision of the competent authorities, allow for the analysis, evaluation and correction, if necessary, of the clinical phage application protocols. A clinical report is compiled for each application, which helps to optimise the phage therapy approaches without the existence of an adapted regulatory frame.

The Czech and Slovak Republics: Stafal®

In the Czech and Slovak Republics, EU Member States, a (publicly reimbursed) anti-staphylococcal bacteriophage product (a phage lysate) is available on the market under the trade name Stafal®[20]. It was approved for market placement by the Czech National Competent Authority, the State Institute for Drug Control. The product is an anti-staphylococcal phage lysate intended for topical treatment of *Staphylococcus* skin infections (registration number 59/0149/89-CS).

Belgium: magistral phage

Faecal transplantation is an established practice in several European countries. Even though faecal matter for transplantation unquestionably meets the Medicinal Product definition, it was not classified as a Medicinal Product in the EU. As such, stool transplants do not need to comply with costly and lengthy development and marketing requirements, such as GMP production and marketing authorisation, and physicians were able to show efficacy in controlled trials[21]. In Belgium, the 'Superior Health Council (SHC)' elaborated and published pragmatic recommendations regarding the therapeutic indications, the procedures, safety and quality of the transplantation of faecal material (Opinion 22 of the SHC). Seen that faecal microbiota contain billions of uncharacterised phages, a Belgian pragmatic solution for phage preparations should logically be possible too.

On 5 July 2016, in response to two parliamentary questions related to the waning implementation of phage therapy, the Minister of Social Affairs and Public Health acknowledged that it is indeed not obvious to deal with phages as industrially prepared medicinal products and therefore suggested exploring the option of magistral phage preparations[22]. The former is subject to constraints related to their production and marketing authorisation, unlike the latter, which was created to offer a practical way to medical doctors to personalise patient treatments to specific needs and to make medicines available that are not (yet) on the market. For instance, allergens and natural hormone combination products, which often lack patent protection, are not produced by commercial manufacturers, but are typically delivered as magistral preparations. In European and Belgian law, a magistral preparation (compounded prescription drugs in the US) is defined as 'any medicinal product prepared in a pharmacy in accordance with a medical prescription for an individual patient' (Article 3 of Directive 2001/83 of the European Parliament and Article 6 quater, § 3 of the Belgian Medicines Law of 25 March 1964). Magistral preparations are compounded by a pharmacist from their constituent ingredients (or under his/her supervision), following the technical and scientific standards of the pharmaceutical art, for a given patient according to a physician's prescription. As a general rule, active ingredients of magistral preparations must meet the requirements of a monograph (describing their preparation) in an official

pharmacopoeia such as the European or the Belgian Pharmacopoeia. However, if no such monograph exists, the Minister of Public Health can still authorise the active ingredients, following a favourable opinion of the national Pharmacopoeia Commission. In addition, magistral preparations may also harbour non-authorised ingredients, providing that they are accompanied by a certificate of analysis. This certificate must be issued by a 'Belgian Approved Laboratory (BAL)', quality control laboratories that have been granted an accreditation by the Belgian regulatory authorities to perform batch release testing of medicinal products. Some BALs belong to the European Official Medicines Control Laboratories (OMCL) network, which groups independent public laboratories that have been appointed by their national authorities. Since there is no 'phage monograph' in any official pharmacopoeia and because of the almost endless variety of phages that could be used as active ingredients and should then each obtain an authorisation issued by the Minister of Public Health, the option of the 'non-authorised ingredient' was chosen. The standard procedure for unauthorised active ingredients only involves the medical doctor, his patient, the manufacturer of the active ingredients, the approved laboratory and the pharmacist, but because of the innovative and very specific character of phage therapy it was decided to also involve the Federal Agency for Medicines and Health Products (FAMHP), the Belgian competent authority for medicines, in the elaboration of the Belgian magistral phage medicine framework. Experts of the Queen Astrid Military Hospital in Brussels, the FAMHP and the Belgian Scientific Institute of Public Health developed a supplier monograph, which describes how phage Active Pharmaceutical Ingredients (APIs) should be produced and tested. It was conceived as an evolving document, applicable to most phages. The phage API monograph received a formal positive advice by the FAMHP on 10 January 2018. As from that date, in Belgium, phages can be delivered in the form of magistral preparations to specific (nominal) patients under the direct responsibility of medical doctors and pharmacists. The general concept of the Belgian magistral phage medicine strategy, depicted in Figure 2, was recently published, including the phage API monograph[22]. Next, standard clinical protocols describing – amongst others – medical indications, formulations and posology for phage applications need to be drafted at the Belgian and at the European level. Ultimately, magistral phage preparations should be listed as products eligible for reimbursement, keeping in mind that their cost price will likely influence the patients' access to phage therapy. Belgian phage preparations have sporadically been 'exported' to France for application in desperate cases, with competent authority approval. Germany is currently investigating the magistral phage pathway and The Netherlands are considering

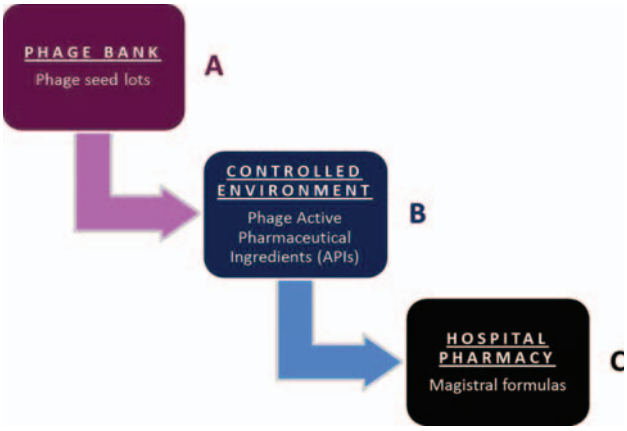

Figure 2. Flowchart of the Belgian magistral phage medicine framework. A) To prevent the unwanted drift of properties resulting from repeated subcultures, characterised phages (phage seed lots) are stored using a tiered banking system (phage bank). B) From a single phage seed lot, a phage Active Pharmaceutical Ingredient (API) is produced according to a monograph. Each batch of phage APIs produced will have a batch record with a detailed description of the production process. A Belgian Approved Laboratory (BAL) performs External Quality Assessments to evaluate the API's properties and quality. C) The phage APIs, accompanied by their batch records and External Quality Assessments, are transferred to hospital pharmacies for incorporation in magistral formulas, by a pharmacist, upon prescription by a physician. Ideally, the most appropriate (active) APIs are selected against the target bacteria (using a 'phagogram') and several phage APIs can be mixed (with a carrier) into one magistral preparation.

the 'importation' of Belgian phage products for clinical trials (personal communication).

Biological Master File

As a European solution to the phage therapy regulatory issues, novel EU regulations based on the 'Biological Master File' (BMF) principle, similar to procedures already existing for chemical drugs, has been suggested[23]. Phage preparations compounded by a pharmacist for an individual patient are not industrially produced and can be regarded as magistral preparations. However, the production of phage APIs, ingredients of these magistral preparations, often fulfils the characteristics of an industrial process. Customised PTMPs are thus somewhere in between magistral formulas and industrially produced medicinal products. This uncomfortable situation should best be addressed within the current EU regulatory framework. The licensing of customised PTMPs could rely on the concept of a BMF. However, the European regulation does not allow an extension of this concept to biological active substances such as phages. Instead, the current registration procedure of biologicals (a Medicinal Product subclass) requires the approval of the Medicinal Product as a whole, not of its active ingredients alone. The BMF concept would thus cover only a part of a biological Medicinal Product application, submitted as a stand-alone package. In the case of customised PTMPs, one BMF for each individual phage, or for a homologous group of phages, could be submitted for licensing by the competent authorities. The BMF

would cover the industrial aspects of the manufacturing process of the phage API and requirements such as a quality module and batch release by a qualified person. In addition, the BMF could also include safety profiling performed for individual phage suspensions. The finished product could be prepared as a magistral formula and would not require approval by competent authorities. This BMF concept could solve a number of regulatory issues with regard to personalised healthcare products in Europe, but unfortunately, extension of this concept to biologicals is not on the agenda of the Commission.

Phages in food and agriculture

Commercial phage products are already used as antimicrobial agents in plant and animal agriculture and food decontamination[24]. These phage-based products navigated easier commercial and regulatory paths than their human therapeutic counterparts. Even though it is expected that these non-human phage applications will pave the way for human applications, we wonder what will be the impact of this type of massive and unlimited environmental use of phages on the emergence of bacterial phage resistance (how fast will it emerge and will it persist or spread?) and on the composition of bacterial populations in the environment? Shouldn't we study this first? Shouldn't we learn from our (antibiotic) mistakes?

P-H-A-G-E.org

Phages for Human Applications Group Europe (http://www.P-H-A-G-E.org) is an international non-profit organisation aiming to support phage research and therapy and to develop a specific regulatory framework for phage therapy in Europe. The organisation was founded in 2009 as a vehicle to explore and help develop a sustainable alternative to the threatening appearance of antibiotic-resistant bacteria. Its members are typically scientists, physicians and specialists in the fields of health economics and legal, regulatory or quality control matters.

Concluding remarks

Awaiting an adapted EU regulatory framework, Member States are exploring national solutions to make phages available to physicians, which are in desperate need of additional tools (in combination with other antibacterials) to fight multidrug resistant infections. Politicians need to support these efforts, preferably at the European level[25]. Phage banks containing well characterised phage stocks (including genome sequences and host specificity) should be set up and this information should be made available to physicians. These phage banks should be able to supply phages for fast amplification and treatment. Even though (European) regulatory approval for use of genetically modified phages is currently non-existent, bio-engineered phages are now gaining interest as future anti-bacterial agent[26].

Conflicts of interest

The authors declare no conflicts of interest.

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Biographies

Dr Jean-Paul Pirnay graduated as Industrial Engineer in Biotechnology at the University College Ghent and obtained a Scientific Degree in Agriculture Development at the University of Ghent. He received his PhD in Medical Sciences from the Vrije Universiteit Brussel. In 1993 he performed his military service and served since then as a researcher in the Queen Astrid Military Hospital (QAMH) in Brussels. He is currently head of the Laboratory for Molecular and Cellular Technology (LabMCT) of the QAMH and research collaborator at the Royal Military Academy. His research interests include human cell and tissue banking, cell therapy, tissue engineering, molecular microbiology and bacteriophage therapy. He was appointed as an Expert to the Belgian Superior Health Council and participated in several National, EU and NATO research task groups and evaluation/advisory committees.

Dr Daniel De Vos obtained a PhD in Sciences (molecular-microbiology), at the Vrije Universiteit Brussel. Professionally he was active in the biomedical field in the tropics (Central Africa, South East Asia) and Europe. He spent 10 years in Central Africa as head of an interdisciplinary team active in the fields of infection, nutrition and epidemiology, with an emphasis on diarrheal diseases and sepsis in malnourished children and antibiotic resistance. He is a visiting professor in microbiology at the Université Catholique de Bukavu (Democratic Republic of the Congo). During 8 years he was project leader for the development of rapid molecular diagnostics, in an industrial setting (Innogenetics/Roche). Since 2006 he is Research Manager at the Laboratory for Molecular and Cellular Technology of the Queen Astrid Military Hospital in Brussels. His actual focus is on infectiology and phage therapy.

Dr Gilbert Verbeken studied at the Ghent University, the KU Leuven and the Royal Military Academy (Belgium). As a biologist, he is more than 30 years active in the field of human cell- and tissue-engineering and tissue banking. Gilbert built up, among other things, significant 'Advanced Therapy Medicinal Products' (ATMP) regulatory experience and is now also involved in the creation of a Bacteriophage Therapy Centre at the QAMH. He obtained his PhD degrees studying the regulatory hurdles and ethical issues related to the (re-) introduction of (natural) bacteriophage therapy in Europe.

The future of phage clinical trials in Australia

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Australia is well positioned to conduct clinical trials in phage-based technology. Despite challenges with translating phage therapy to mainstream medicine, our regulations are designed for safe and innovative development. Recent success indicates that Australia is ideal for conducting further phage clinical trials. There are also expert clinical research organisations and generous tax incentives.

Historically, there have been barriers to translating phage therapy from 'bench to bedside'. Some strategies for broader acceptance of phage therapy have been evaluated[1–3] and the industry consensus is to gather quality clinical evidence regarding safety, tolerability, and efficacy[2]. As such, clinical trials (CTs) are a critical interface for translating phage therapy. The components that can influence the success of a clinical trial are depicted in Figure 1.

More broadly, the translation of phage therapy can be broken down into three main components: the phage, the CT design, and the regulations. There is ongoing debate regarding the ideal strategy for translating phage technologies. Some researchers suggest that instead of using natural phage cocktails (a mixture of numerous strains of phage targeting the same host bacterium), phage-based products such as lysins and depolymerases may be used as an alternative (and simpler) pathway to regulatory compliance[4,5], while other researchers argue that, in fact, regulations should change[5,6]. Instead, the author proposes that the main solution to translating phage therapy is to change the CT design. This article summarises: some phage technologies; recent phage CT outcomes, the potential design for other phage-related CTs; and the process of conducting a phage CT in Australia.

Phage cocktails

Phage cocktails, rather than individual isolates, are often used to treat recalcitrant biofilms rather than individual isolates because they broaden the range of susceptible hosts and reduce the risk of replacing treated hosts with phage-resistant mutants[7,8].

However, phage cocktails often raise more regulatory flags. Phage replication can result in mutant phages and there are concerns regarding potential genetic transfer of pathogenic elements, such as shiga toxin to bacteria. High-throughput sequencing may be an effective tool used during a CT to collect phage genetic data[9] and enable monitoring of mutations while informing clinical researchers and regulators. In addition, this may assist with collecting pharmacokinetic and dose finding data[10]. Others suggest that genetically modified phages (GM-phages) may control for random genetic transfers and mutations, thus improving the chances of regulatory success.

Genetically modified phage

Over the past 30 years, phages have proven to be genetically-malleable tools[11–15]. New developments using genetic engineering (a.k.a. synthetic biology) to create phages[12] may be relevant to the future of phage CTs. Some researchers compare GM-phages to genetically-programmable machines[16] and, if gene switches are inserted, they may also reduce risks associated with uncontrolled gene transfer[15]. In 2018, the Nobel Prize in Chemistry for was awarded to three scientists for their work in 'phage display of peptides and antibodies'[17,18] and 'directed evolution'[19]. Some researchers argue that genetic engineering of phages may secure intellectual property and in doing so increase the potential for funding CTs. Moreover, since the discovery of CRISPR/Cas9 there has been a rapid increase in the number of technologies using GM-phages[20]. Functions including conditional expression, conditional replication, and non-integration can be included. Although it is yet to be demonstrated, such innovation may improve the chances of regulatory approval while also providing an opportunity to create new industry for Australia.

Phage clinical trials

In the recent past, many phage therapy CTs have used traditional fixed designs and methods that have not been able to adapt their trial parameters to improve scientific precision and provide groundwork for subsequent trials. In 2009, results from a British phase I/II CT demonstrated efficacy using a single dose of a topical

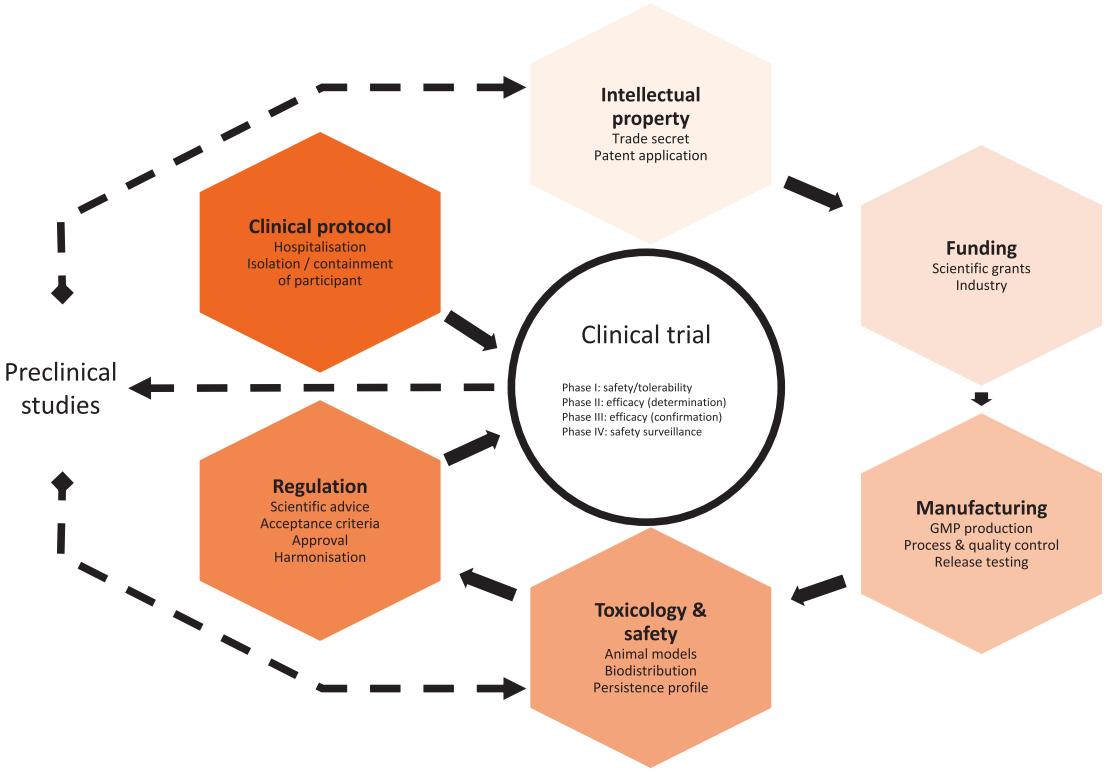

Figure 1. Essential steps in the translation of viruses in the clinics[8].

six-phage cocktail for antibiotic-resistant *Pseudomonas aeruginosa* in patients with chronic otitis. Unfortunately, many of the patients had recurrence of their infection. The authors recommended another randomised, double-blind CT with multiple doses but this trial never occurred[21].

In 2017, researchers from The University of Adelaide presented promising results from a landmark phase I CT evaluating the safety and tolerability of an intranasal irrigation containing a three-phage cocktail[22] targeting *Staphylococcus aureus* in patients with surgically-recalcitrant chronic rhinosinusitis[23]. Safety, tolerability, and efficacy were demonstrated, and like the otitis trial, future recommendations included longer duration of the phage therapy.

In 2019, results from the European PhagoBurn CT were published. This was the world's first phage CT to be performed according to Good Manufacturing Practice and Good Clinical Practice. This multi-centre, randomised, controlled, double-blind phase I/II CT demonstrated tolerability of a 12-phage cocktail targeting *Pseudomonas aeruginosa* burn wound infections but was unable to demonstrate efficacy. Participant recruitment was slower than expected because many of the screened participants had polymicrobial infections. In addition, the bacteria isolated from participants who failed phage therapy were resistant to low phage doses. Therefore, the authors recommended increased phage dose in future to demonstrate efficacy without allowing the opportunity for phage-resistant mutants to develop[24].

Adaptive design

Adaptive CT design[25] may have advantages over the aforementioned traditional fixed randomised, controlled design for further development of phage technologies. Adaptive CT designs are less rigid and allow for adjustment of components such as dose, frequency, duration, allocation to different treatment arms, and sample size during the CT. The statistical modelling for adaptive CTs generally use a Bayesian inference continuous reassessment approach[26], which can create updated distributions as the number of participants increases. In doing so, valid real-time data can be obtained from the smallest possible sample size without exposing participants to unnecessary risks. One drawback of this approach is that preparation of an efficient adaptive CT design may require multidisciplinary input and the use of simulation software[27]. Whilst this would increase lead-up costs, it often results in overall cost reduction and is an effective approach to flagging trial related issues in advance. Ultimately, adaptive designs can evolve to suit the dynamic nature of phage therapy CTs. They may also be useful in evaluating other GM-phage applications such as anticancer and gene-based therapy phage products.

Regulatory process in Australia

In Australia, the Therapeutic Goods Administration (TGA) has adopted many of the European Medicines Agency policies. Both natural phage and GM-phage are considered 'gene transfer

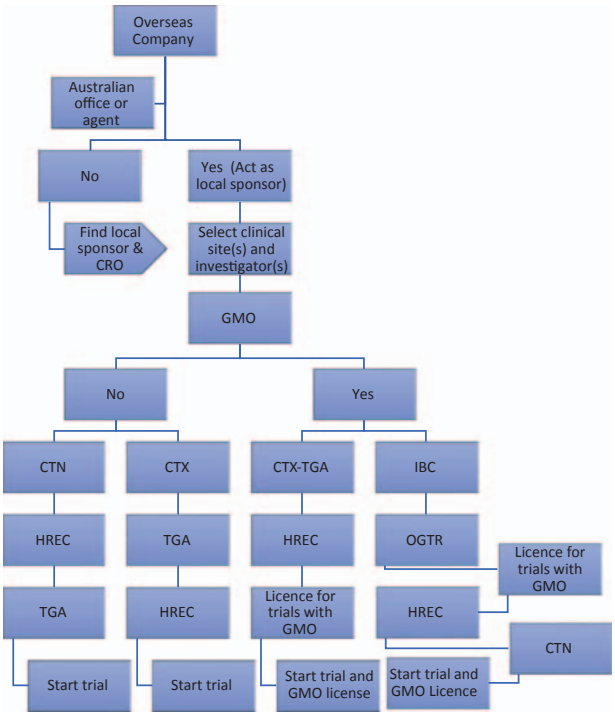

Figure 2. How to start a clinical trial in Australia for unregistered products. Adapted from LSQ (2016) Starting a Clinical Trial[12]. CRO, Contract Research Organisation; GMO, Genetically modified organism; CTN, Clinical Trial Notification; CTX, Clinical Trial Exemption; IBC, Institutional Biosafety Committee; HREC, Human Research Ethics Committee; TGA, Therapeutic Goods Administration; OGTR, Office of the Gene Technology Regulator.

biological medicines'. The Australian regulatory process for both natural and GM-phage is shown in Figure 2[28].

There are currently no Australian-owned phage therapy companies. For CT to be undertaken in Australia, the law mandates that an overseas company must have a local representative, also known as a sponsor (a person or entity who resides in Australian and either imports, exports, or manufactures therapeutic goods). The person or entity who is in this role has primary responsibility for many of the decisions made during the planning and execution of the CT. The local sponsor will then review and select the appropriate clinical site(s) and investigator(s). The subsequent regulatory pathway will then depend on whether the phage is genetically modified. The sponsor can confirm this based on the Gene Technology Act[29] and the Gene Technology Regulations[30].

If the phage (product) is not a result of genetic modification, the sponsor will then decide whether it should pursue the TGA's Clinical Trial Notification (CTN) scheme or the Clinical Trial Exemption (CTX) scheme. This should be done in collaboration with the Human Research Ethics Committee (HREC) and will depend on whether the HREC has appropriate scientific and technical expertise to assess the safety and efficacy of the product. The CTN scheme is often viewed as efficient and cost-effective compared to the comprehensive review required via the CTX pathway. However, one benefit of the CTX pathway is that once a CTX application is approved, the sponsor may conduct any number of CTs under that application without further assessment by the TGA. For any CT, a HREC evaluates whether the risk-benefit ratio is favourable for the participant. To improve the potential benefits to the participant, the product should target a disease of unmet need and have demonstrated safety and efficacy in preclinical studies. In order to make a reasonable assessment, the HREC evaluates at least three essential documents: investigator brochure, trial protocol, and patient informed consent form. The investigator brochure should include both phage-relevant data[8] and published studies that support validity (e.g. randomisation, sample size calculation, and blinded outcome assessments[31,32]).

Should the product be a genetically modified organism (GMO), then an alternative pathway will be required. A GMO must first be evaluated by an approved Institutional Biosafety Committee (IBC) which will determine the risk of GMO release into the environment and the suitability of the licence applicant to be accredited under the Gene Technology Act. Although this is an additional requirement, Australia's regulators do not duplicate evaluations and the National Gene Technology Scheme[33] ensures safety without stifling innovation[33].

Once the Office of The Gene Technology Regulator issues a licence to the sponsor, the IBC and HREC will recommend either the CTX or CTN scheme to the TGA. This is different to the natural phage pathway and serves to eliminate regulatory duplication. Additionally, if the product is manufactured overseas, an import permit from the Department of Agriculture and Water Resources may be required. This can be evaluated through the Biosecurity Import Conditions website tool[34]. Participant recruitment can then commence once all appropriate approvals are obtained.

Why Australia?

Australia is considered attractive to international sponsors wishing to conduct CTs because of key financial and logistical frameworks. The research and development tax incentive scheme provides up to 43.5% reduction in a company's income tax liability and given that the average phase I CT costs greater than $2m, the potential savings are significant. In 2013, Australia setup The National Mutual Acceptance Scheme, which is a Memorandum of Understanding between most states and territories to allow for 'once only' scientific and ethical review for multi-centre CTs conducted at public health organisations. This improves efficiency by reducing duplication. Readers can review the numerous clinical trial documents that the Australian government has provided online[35–37].

The translation of phage therapy requires an adaptive approach. Out of the three components, CT design is easier to adjust than the

phage, and much easier than the regulations. For the phage-therapy industry to reposition itself from 'controversial' and 'fringe' to mainstream, judicious use of resources in high yield trials that adapt to confounding factors should be prioritised. Nonetheless, with collaboration and experienced investigators, these hurdles can be minimised and Australia can establish itself as a good choice for phage CTs due to high quality infrastructure, efficient regulators, and the research and development tax incentive.

Conflicts of interest

The author declares no conflicts of interest.

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Biography

Dr Keith Potent is an ENT registrar (The Tweed Hospital), a Principal Investigator (Griffith University Clinical Trials Unit), and a PhD (Translational medicine) candidate (Monash University). His research interests are the translation of phage technologies and Ear, Nose, and Throat surgery.

Bacteriophage therapy for severe infections



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The rise of multiple antibiotic resistance in clinically relevant bacteria has created a global crisis with increasing burden on healthcare systems. The need to optimise alternative therapies to antibiotics, particularly in high risk nosocomial settings, is therefore immediate. Bacteriophages are specialised lethal viruses of bacteria, and an underused clinical resource for the treatment of severe infections refractory to antibiotics. Both the gaps in knowledge of bacteriophage biology, particularly the details of host-pathogen dynamic interactions, and legislative hurdles related to the regulation of natural microorganisms for therapy have delayed progress in bacteriophage clinical applications. At the Westmead Institute for Medical Research (WIMR), in collaboration with Westmead Hospital (Western Sydney Local Health District, WSLHD) and the University of Sydney (USyd), we have been investigating rational design protocols for routine bacteriophage application in clinical practice and testing bacteriophage therapeutics on patients suffering from multidrug resistant (MDR) severe infections.

Bacteriophage therapy

Brief introduction

Bacteriophage (phage) therapy exploits the natural predator-prey interaction between phages and their exclusive targets, bacteria, and involves the use of purified mixes of multiple viruses (cocktails) to directly administer to patients. Only lytic phages, which replicate exponentially inside bacteria immediately after infection, are considered appropriate for therapy due to their reduced transduction potential[1,2]. To date most of the characterised natural phages (95%) are double-stranded DNA, tailed viruses belonging to the order Caudovirales, which are readily isolated from most environmental sources (soil, water, animal faeces, etc.)[3]. Their highly effective lytic activity is based on two main mechanisms: specific recognition of complementary receptors on the host cell surface, and bacterial cell lysis at the end of virion (phage progeny) replication leading to selective pathogen eradication[4]. Due to this unique interaction between bacterial receptors and phage anti-receptor structures, most phages have a narrow host range that can be considered advantageous for the development of targeted therapy and for the lack of collateral damage to the resident human microflora[4,5], with much of the renewed commercial interest in phage applications centering around this target specificity.

The discovery of phages dates back to more than a century ago and is ascribed to both an English physician (F. Twort) and a French-Canadian microbiologist (F. d'Herelle) who independently observed and reported the lysis phenomenon caused by bacteriophage activity[1,2,6]. However, it was d'Herelle alone who, as early as 1919, pioneered the successful clinical application of phages to treat infections in humans[2,6]. Yet mixed clinical outcomes along with the discovery of broad-spectrum antibiotics in the early 1930s meant that phage therapeutic application all but ceased in Western medicine[1,7]. Conversely, phage research and applications continued unabated in the former USSR, particularly in two main research centres: the Eliava Institute of Bacteriophage, Microbiology, and Virology of the Georgian Academy of Sciences (Tbilisi, Georgia) and the Hirszfeld Institute of Immunology and Experimental Therapy of

the Polish Academy of Sciences (Wrocław, Poland), where phages have been continuously used in preclinical and clinical treatment of common infections since the first half of the 20th century[1,2,8]. However, much of the accumulated experience in these countries has been anecdotal with insufficient (qualitative rather than quantitative) or inaccessible clinical records.

Regulatory framework

Phages are natural organisms, arguably the most abundant life-form on Earth[9]. They have evolved closely and dynamically with their bacterial host and are therefore specific and effective in selectively eliminating their target[3]. They have low environmental impact and have shown to have no serious side effects on bystander microorganisms[2,5,7]. They are self-replicating in the presence of their target, facilitating dosing regimens[1,2,7], and have been successfully employed to treat even MDR infections[2,7]. So, why isn't bacteriophage therapy routinely employed in the clinic yet? There are in fact a number of unresolved issues, including biology-related knowledge gaps in resistance development, transduction potential, immunogenicity, host range mechanisms, and penetrance, as well as regulatory hurdles associated with the lack of both robust scientific protocols able to withstand the scrutiny of Western regulatory agencies [e.g. Therapeutic Goods Administration (TGA, Australia), Food and Drugs Administration (FDA, US)], and of appropriate legislation for the commercialisation and use of natural organisms as therapeutics[1,10]. How phage therapy can be best integrated into established clinical models of drug development, pharmacokinetics and pharmacogenomics, and associated regulatory schemes remains a challenge[11,12].

The use of phages and phage-based enzymes in the EU and US is currently permitted through experimental therapy only and subject to Article 37 of the Helsinki Declaration[13–15]. Only recently (2006) the FDA has recognised the designation of phages as 'generally regarded as safe', allowing for the use of phage in clinical practice and opening the road towards the implementation of *bona fide* clinical trials. Both the TGA and FDA define Good Manufacturing Practice (GMP)-produced phage cocktails as investigational drugs, subject to laws and regulations for this category set by each agency. In the EU, Belgium has been at forefront of progress in the regulation of phage therapy for routine clinical practice by implementation of a 'magistral phage medicine strategy' with magistral (Article 3, Directive 2001/83 and Article 6 quater, § 3 of the Law of 25 March 1964) phage products approved for personalised patient therapy[16].

A number of phage therapy phase I and II initial (small sample sizes) trials has been conducted in recent times[10,17]. Although phage-based products have received FDA licensure for food safety applications, no licensed phage product prepared under GMP for infection treatment has yet reached the Western market[11]. The current practice for stable (prolonged shelf-life) and safe (LPS-purified GMP produced) phage cocktail preparation for therapy requires the collaboration of commercial entities and research labs[2]. A decade ago only a handful of companies specialised in bacteriophage products[7,12]. Currently bacteriophage research and development is experiencing a veritable renaissance with several new commercial enterprises established worldwide[18].

Working with bacteriophage

Bacteriophages (Pyophage #051007, Eliava Institute, Georgia) were successfully trialled at Westmead Hospital more than 10 years ago under 'compassionate use' guidelines (TGA) on a patient suffering from a refractory *Pseudomonas aeruginosa* urinary tract infection[19]. Following this, a series of projects were aimed at both optimising the rational design of phage cocktail preparation protocols, and implementing phage therapy in critical care settings, through national and international, research and industry collaborations. A study in Adelaide showed that self-administered phage-based nasal washings (AB-SA01, AmpliPhi Biosciences Corporation) were a safe and likely effective treatment for chronic staphylococcal sinusitis[20] and, in late 2018, we reported the first intravenous use of the same product for severe sepsis control in Westmead[21].

Research

Considering the many areas of phage biology that require better understanding, a rational design approach is critical for the optimisation of phage cocktail preparations for effective and long-lasting therapy[2]. Therefore, in conjunction with clinical efforts, we are seeking to establish a rationalised phage cocktail preparation protocol (Fig. 1) applicable first, as proof-of-concept, to the eradication of highly virulent MDR clones (e.g. ST131 *Escherichia coli* and CG258 *Klebsiella pneumoniae*; NHMRC 1107322). Access to a large well curated collection of clinical isolates has allowed for the selection and full characterisation of exhaustive target bacterial populations, while phages were sourced both from an existing library (available through research collaboration with AmpliPhi Biosciences Corp.) and *de novo* isolation from environmental reservoirs.

Host range testing, matched with detailed genomic analyses of both viruses and bacteria, reveals the unique specificity of phage candidates towards the chosen targets, allowing for careful selection of optimal therapeutic cocktails. *In vivo* work including, but not limited to, murine models (e.g. for ST131 *Escherichia coli* and CG258 *Klebsiella pneumoniae* gut colonisation and severe bacteraemia models; NHMRC 1107322), must also be performed in order to define *in vivo* dynamics, resistance development potential and evolution trajectories for each bacterial population/best-specific-cocktail combination. In an effort to streamline this process towards therapy design for multiple sepsis-causing nosocomial pathogens, high-throughput susceptibility assays and host range manipulation strategies are also an essential requirement.

Clinical experience

In 2018, the AB-SA01 GMP phage preparation (AmpliPhi Biosciences Corp.) was used in the treatment of severe *Staphylococcus aureus* infection in humans at Westmead (Sydney, NSW). The

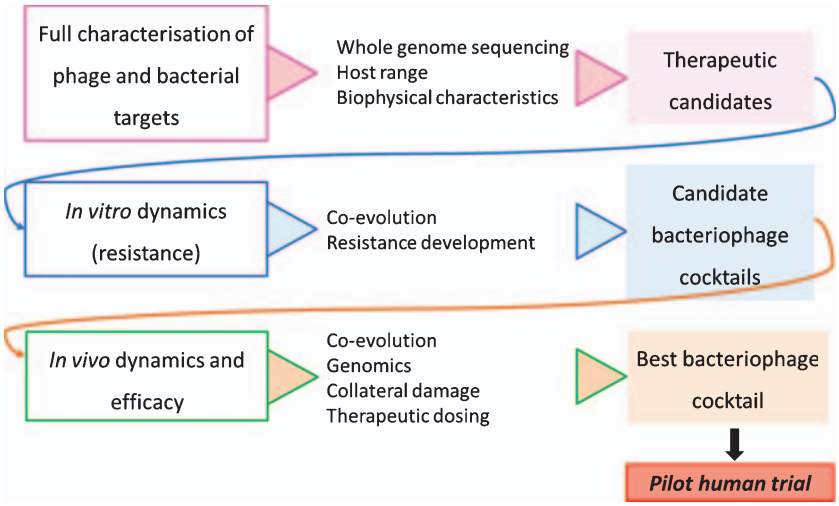

Figure 1. Rational design protocol for the preparation of 'best' (most effective, least resistogenic) therapeutic cocktails.

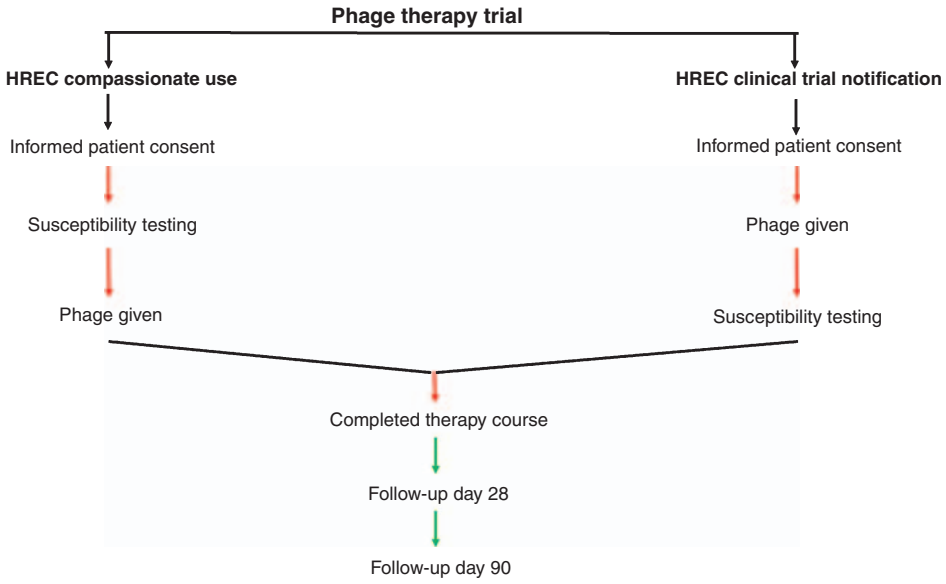

Figure 2. Study outline for treatment of *S. aureus* severe infection using a GMP bacteriophage cocktail. The differently coloured arrows indicate the different steps in the clinical protocol: black, ethics; red, treatment; green, post-treatment follow-up.

Westmead experience with intravenous administration of adjuvant bacteriophage to critically ill patients being treated for severe staphylococcal disease, including prosthetic valve endocarditis, was reported at the Infectious Diseases Society of America (IDSA) annual scientific meeting in late 2018[21]. In Australia, *S. aureus* infections cause ~20–25% of lethal septic shock, and at Westmead Hospital >100 unique sterile-site isolates are identified each year. In our single site investigator-initiated study, participants were recruited under HREC (Human Research Ethics Committee, WSLHD and WIMR) approval. The phage cocktail used in this work is currently available under the US FDA's Expanded Access regulations (http://clinicaltrials.gov)[22]. Critically ill patients with severe *S. aureus* infection were enlisted for the study under the TGA Special Access Scheme (18 May 2017 onwards) and subsequently under the TGA Clinical Trial Notification (CTN) scheme (from 6 July 2018). It is here noteworthy that the HREC allowed for CTN with *ab initio* bacteriophage administration after review of interim safety data from the first set of recruited patients (Fig. 2). The devised protocol prescribed treatment with phage in conjunction with standard antibiotic therapy and 90-day follow-up to define microbial kinetics as well as clinical outcomes. Treatment was reported to be associated with reduction in bacterial burden and with no adverse events[21].

It is expected that the recent clinical experiences in Australia and overseas will pave the way for Phase II and III controlled randomised trials for this and other phage products at various sites. Future carefully designed controlled studies are expected to commence in 2019.

Finally, at Westmead we are working toward the development of bacteriophage bio-banking and linked patient sample collections as a state-wide resource available for all pathogen researchers in NSW, with the aim of implementing sustainable national and international networks. We are active supporters of the newly established ASM Bacteriophage Biology and Therapeutics Special Interest Group (SIG) (https://bacteriophagesig.blogspot.com/), aiming to

promote bacteriophage research in Australia and to connect phage researchers and any others who have an interest in this field.

Conflicts of interest

The authors declare no conflicts of interest. CV, APF and RCYL are not employees of AmpliPhi BioSciences and do not own any shares.

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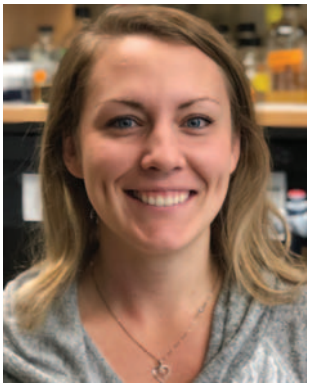
Biographies

Dr Carola Venturini is a research microbiologist whose work has primarily focused on the role of mobile genetic elements in the evolution of infectious bacteria. Her work has a multidisciplinary approach that combines traditional microbiology molecular methods with bioinformatics (genomics). Since 2013, Carola has been part of Prof Jon Iredell's Bacterial Pathogenesis research group at The Westmead Institute for Medical Research (Sydney, NSW) leading as project manager applied research investigating the ecology of the gut microbiome related to mechanisms of antibiotic resistance in the Enterobacteriacae and exploring the use of bacteriophage in combating infective multidrug resistant bacterial clones of clinical relevance. Carola is the NSW-representative executive committee member of the ASM Bacteriophage Biology SIG.

Dr Aleksandra Petrovic Fabijan obtained her Bachelor, Master and PhD in Biology at University of Novi Sad (Serbia). During her Master Thesis she studied antibiotic resistance of *E. coli* isolated from digestive tracts of wild birds. She completed her PhD in Microbiology in 2016 investigating *Bordetella bronchiseptica* specific bacteriophages and their antimicrobial potential. During her PhD studies she also investigated alternative antimicrobial agents against multi-resistant *Acinetobacter baumannii* and *Helicobacter pylori* strains. Aleksandra recently joined Iredell's team as a Phage Biologist and her work is focused on bacteria-bacteriophage interaction and host response in septic patients receiving adjuvant bacteriophage therapy.

A/Prof Ruby Lin joined the Iredell lab at The Westmead Institute for Medical Research at the end of October 2017 as project manager, after a short stint in industry. She is the scientific lead for an investigator-led clinical trial involving treatment of severe staphylococcal infections using bacteriophage therapy. Her research focus has been microRNA driven dysfunctions in eukaryotic disease model systems including mouse/rat models and humans. She was named NHMRC Peter Doherty fellow (2005–8) and UNSW Global postdoctoral fellow (2009–14). She is a conjoint Associate Professor at the UNSW. During her presidency at Australasian Genomic Technologies Association (AGTA), a prominent society in genomics in Australia and NZ with members from industry and academia, she implemented gender equality at its annual meetings. She is heavily involved in promoting gender balance and women in STEM through various professional networks.

Sourcing phages for compassionate use



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Antibiotic resistance is a phenomenon that knows no geographical borders, so addressing this crisis is a worldwide public health priority. While total global resistance rates are difficult to estimate and vary between countries, an international report asserts that the development of new antibacterials is essential to ensuring the future ability to treat bacterial infections[1]. Bacteriophage (phage) therapy is a likely contributor to resolving potentially devastating effects of antibiotic resistance, yet no phage product currently holds a marketing authorisation that would permit their free use in clinical medicine outside of former countries of the Soviet Union, where phage therapy is a long-standing practice[2,3]. In the interim, the compassionate use of phage therapy (cPT) remains a possible treatment avenue for cases of antibiotic failure, and several competency centres, physicians, and researchers have achieved therapeutic benefits with this option. As antibiotic resistance continues to rise, there is much to be done in order to streamline cPT efforts, particularly in terms of phage sourcing, in order to reach more patients in an efficient, effective, and safe manner. This article highlights how cPT can be coordinated, and describes the experience of cPT in Australia.

Compassionate treatment denotes the use of unapproved medicines outside of clinical trials for the treatment of patients for whom approved therapeutic options have been exhausted unsuccessfully. The premise of compassionate use is stated in Article 37 of the 'Helsinki Declaration of Ethic Principles for Medical Research Involving Human Subjects', an international guideline for research on human subjects[4]. Both Articles 3 and 37 concern individuals at the end of their therapeutic options. Official oversight of compassionate use programs varies from country-to-country, but is handled by regulatory agencies, such as the FDA in the USA or the EMA in the European Union[5–7]. In Australia, access to unapproved therapeutic goods can be attained through a Special Access Scheme (SAS), which is divided into three categories (A: notification for the seriously ill; B: application; C: notification of use of specified products)[8]. The Second Australian report on Antimicrobial Use and Resistance in Human Health has identified 14 target pathogens for which resistance rates to certain antibiotics varied widely from <1 to 96% of isolates, indicating an existing need for alternative treatment options, such as cPT[9].

The use of phage for compassionate treatment of antibiotic-resistant infections is increasing, with >20 published papers from experimental treatment centres, pilot studies, and case reports since 2000, reviewed in[10–15]. A diversity of infections have been treated by cPT, including endocarditis[16], diabetic toe ulcers[14,17], abdominal cysts[18], prostatitis[15,19,20], otitis[15] and osteomyelitis[12], encompassing both local and systemic routes of administration. One case report described the treatment of a refractory urinary tract infection caused by a multidrug-resistant strain of *Pseudomonas*

aeruginosa in Australia[21]. Administration of a six-phage personalised cocktail directly into the bladder resulted in sterile urine cultures for this pathogen after only eight days (although antibiotics were administered six days after phage therapy started and may have contributed to the final effect). The case required the cooperation of international researchers and physicians from Australia, France, and Georgia to obtain therapeutic phages and to coordinate treatment. For this treatment to be possible, phages were prepared by the renowned Eliava Phage Therapy Center in Tbilisi and sent on-site. This is one of the few studies to date that documents multiple aspects of human treatment that are critical for understanding phage therapy, including viable phage and bacterial counts and phage sensitivity testing, and should serve as an example for future reporting criteria[21,22]. This case, like others, highlights the need for close collaboration between physicians and phage researchers, sometimes at an international level, in order to provide know-how and facilitate access to therapeutic phages for compassionate means until approved alternatives become available.

Finding phages for cPT

Access to phages with activity against the patient's bacterial isolate is evidently essential for compassionate use, and while phages are ubiquitous in nature, environmental isolation requires starting the phage selection process from scratch and having sufficient infrastructure and resources. Given the high level of fundamental and translational research conducted on phages, many phages suitable for cPT already exist in both academic and state research institutions. The phage community is a highly cooperative and supportive research area; many of its members have voiced their willingness to share both their experience and their phages for cPT cases, and several have already done so. Another source of phages for compassionate use are small biotech companies that are in the process of developing clinical-grade phage products, such as Pherecydes Pharma in France, Adaptive Phage Therapeutics in the USA, and Ampliphi Biosciences, which is based both in the USA and Australia.

An initiative to organise sharing for cPT, called Phage Directory, was founded by two of the authors in 2017[23]. This organisation was created in response to a need for *Burkholderia cepacia* phages for the authorised cPT treatment of a 25-year-old cystic fibrosis patient in Pittsburgh, PA (USA); the lack of readily-available phages delayed cPT and the patient passed away. Currently, the academic phage laboratories and phage banks registered on Phage Directory represent more than 20 different countries, including labs at Monash University and the University of Adelaide in Australia, and contribute phages that target >30 host genera. An example of how the Phage Directory network has facilitated phage sharing for cPT is presented here (Figure 1).

Figure 1. Community-sourcing of phages for cPT through Phage Directory. Graphical representation of the global response to a Phage Directory request for *Klebsiella pneumoniae* phages for a patient suffering from an antibiotic resistant infection in Helsinki, Finland in 2018.

In late 2018, Phage Directory coordinated the sourcing of *Klebsiella pneumoniae* phages for a patient in Helsinki, Finland by sending an electronic alert to its network of registered labs and phage collections. This was in response to a request from a phage laboratory in Finland that was working with the patient's medical team. Within one week, nine academic labs and one phage biotech had offered to test or share phages, and within three weeks, more than 175 *Klebsiella* phages had been tested (Figure 1). At least six of these phages were found to be active against the patient's isolate *in vitro*. This process is ongoing; phages have not yet been administered to the patient, as appropriate preparations are currently being made. This example illustrates the willingness of the phage community to participate in such efforts and shows how phage sharing can be expedited through central coordination.

cPT in Australia

After the published case from 2011, no cPT uses were reported in Australia until 2017. Ampliphi Biosciences then announced the intravenous administration of their phage preparation against *Staphylococcus aureus*, AB-SA01, for an endocarditis infection under Category A of the SAS framework[8,24]. Since then, the company has established an expanded access agreement to compassionately use AB-SA01, and another product against *P. aeruginosa*, in collaboration with Western Sydney Local Health District and Westmead Institute for Medical Research[25]. They have now reported treating 13 patients suffering from serious *S. aureus* infections with an 83% success rate[26]. While these data have not yet been formally documented in peer-reviewed publications, they have been publicly presented both at scientific conferences and as press releases. The AB-SA01 product was previously tested in phase l clinical trials for topical administration and for chronic rhinosinusitis, and it is likely that the positive phase l data, along with positive results in cPT, will support phase ll trials that could lead to marketing authorisation for this product.

Conclusions

The ultimate future of phage therapy awaits the completion of randomised, controlled clinical trials in order to determine efficacy and attain marketing approvals. However, as there is no definitive date for when this may be accomplished, compassionate treatment options are an impactful way to address the clinical needs of patients suffering from intractable antibiotic-resistant infections today. In addition to the instances in Australia, cPT is also occurring around the world at phage therapy competency centres such as the Phage Therapy Unit at the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy in Poland[27–30], the Eliava Phage Therapy Center in Tbilisi, Georgia, and the Center for Innovative Phage Applications and Therapeutics (IPATH) at the University of California San Diego School of Medicine[10,18,31]. cPT is also being done by independent medical teams through different access schemes in the USA, France, and Belgium[32], and academic phage labs and biotech companies around the world are providing phages on behalf of patients. Collectively, this indicates an international inclination to support cPT. It is the hope of the authors that by streamlining the process of accessing and sharing therapeutic phages, cPT will be available to more patients in need.

Conflicts of interest

Two of the authors, Jessica C Sacher and Jan Zheng, are co-founders of Phage Directory.

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Biographies

Jessica Sacher completed her PhD at the University of Alberta, where she studied the interactions between the gut pathogen *Campylobacter jejuni* and its phages. In 2017, she co-founded Phage Directory as a way to help foster collaboration between medical and industry professionals, researchers, and regulators to accelerate phage research and its translational applications.

Jan Zheng is a product designer with a background in computer science, psychology, with a Master's degree in Human-Computer Interaction from Carnegie Mellon University. In the past, he has worked with companies like Coca-Cola, Microsoft, and L'Oreal, to design and build better digital products and user experiences. He co-founded Phage Directory to apply his background in UX design and web engineering to make a difference in the areas of health and phage technology.

Shawna McCallin is a postdoctoral researcher at University Hospital of Lausanne (CHUV), Switzerland who has previously been involved in two phage clinical trials for *E. coli* diarrhea and *S. aureus* carriage. She has extensively studied commercial phage preparations from Russia and Georgia, with a focus on *S. aureus* phage diversity. Recently, she is developing a phage sensitivity assay for on-site testing and expanding her phage research to the microbiome.

Phage research in 'organ-on-chip' devices

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The use of 'organ-on-chip' devices in microbiology research presents enormous opportunities for fundamental and translational research[1–4]. Yet these approaches have not been widely embraced by the microbiology field. This is particularly evident with bacteriophage (phage) research applications. Traditionally phage research has been an early adopter of experimental techniques and approaches[5], having catalysed research in biotechnology, environmental biology, sequencing, and synthetic biology. Here we discuss some of the opportunities that organ-on-chip devices present to both phage and microbiology research, and provide a 'how to' guide for researchers interested in utilising this approach.

'Organ-on-chips' are micro-engineered biomimetic devices that replicate key functions, activities and physiological responses of entire living organs[6]. The approach has been used to develop beating hearts[7], simulate breathing lungs[8], sustain a gut microbiome[3,9] and even develop interconnected neurons of the brain[10]. Devices are typically micro-fabricated to contain channels that are lined with cultured human cells, which mimic organ-specific architecture and functions *in vitro*[6]. The device structure varies depending on the organ of interest. For instance, the gut-on-chip can comprise of a single[4] or double channel structure[9], with channel dimensions varying between 500–1000 µm wide and 150–250 µm high. The single-channel gut-on-chip forms the simplest structure, being enclosed by a glass slide upon which a layer of gut epithelial cells is grown. In comparison, the double-channel gut-on-chip is constructed by joining two single-channel devices together with a thin porous membrane separating the two channels. The membrane supports the gut cell layer within the top channel while the bottom channel represents the vascular system of the gut.

The fabrication, operation and experimentation of organ-on-chip devices typically require the convergence of numerous fields including engineering, cell biology and microbiology; presenting a high technical barrier for research applications. Yet overcoming these challenges allows us to probe the interactions between phages, their bacterial hosts and 'life-like' organs to answer therapeutic, ecological, and fundamental questions. For example, a mucus-producing lung-on-chip model was used to describe phage adherence to mucus layer, thereby forming a non-host-derived barrier against bacterial infection[4]. Other studies have demonstrated the maintenance of a gut microbiome and Coxsackie virus infection using a gut-on-chip model[3,9]; approaches that can be modified to investigate gut phage-bacteria interactions. In essence, the organ-on-chip provides researchers the benefit of *in vitro* amenability while experimenting with phages under biologically relevant conditions.

The organ-on-chip in four steps

Step 1: designing the organ-on-chip mould

The first step to creating an organ-on-chip is to fabricate a mould. Two commonly used options are photolithography and 3D-printing. Photolithography (Figure 1) is commonly used in engineering fields, but is technically challenging; requiring specialist equipment and reagents. However, this technique is virtually limitless in creating complex designs at the nanoscale[11]. The technique starts with depositing a photosensitive polymer on a substrate. By controlling ultraviolet (UV) light exposure on the substrate, the polymer will polymerise to the desired feature pattern, which is subsequently developed by washing away soluble unpolymerised regions.

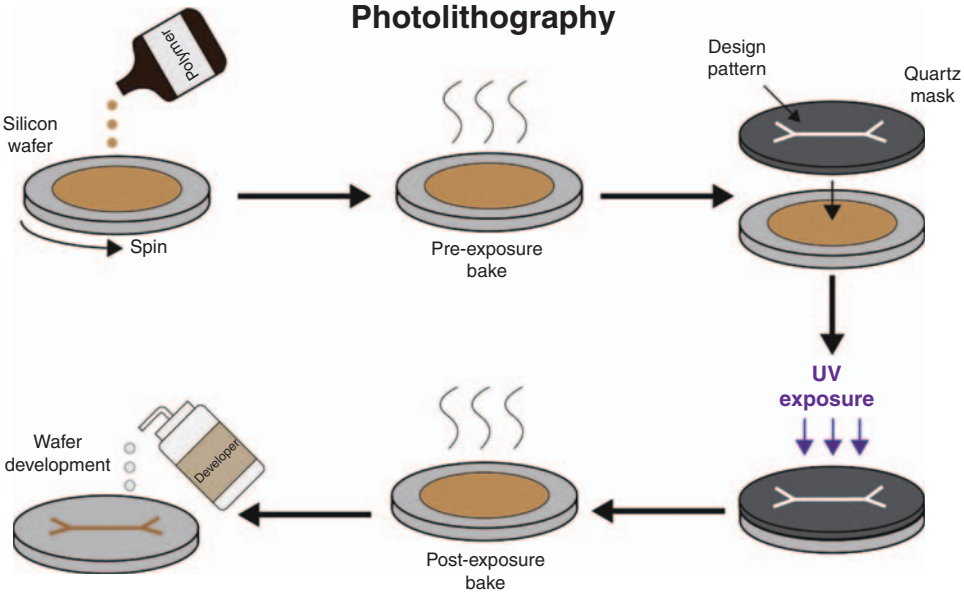

Figure 1. Organ-on-chip mould fabrication using photolithography. In this process, a photosensitive polymer (SU-8 is commonly used) is deposited onto a silicon wafer, baked, and overlayed with a quartz mask containing the desired features of the device. Exposure to UV light polymerises and solidifies the polymer to create the mould for subsequent use.

Figure 2. 3D printing the organ-on-chip mould. The mould is drawn using a modelling software, such as SolidWorks®, then converted to a 3D printer-readable file (.STL format) and sent to a 3D printer for device printing.

Alternatively, 3D-printing (Figure 2) offers a much quicker, easier, and cheaper route to fabricate organ-on-chip moulds. However, unlike photolithography, 3D printing has a much lower printing resolution, typically in the micrometre scale[12]. Nonetheless, the accessibility and speed that 3D-printing offers enable researchers to quickly create simple organ-on-chip moulds for subsequent manufacturing, set-up, and experimentation[11].

Step 2: making the organ-on-chip

Once a mould is obtained, a variety of materials can be used to manufacture organ-on-chip devices. However, none has matched polydimethylsiloxane (PDMS) for its advantages in biocompatibility, permeability to gases, optical transparency and material flexibility[13]. In addition to its advantages in biological applications, fabricating with PDMS is fairly straightforward (Figure 3) and does not require special expertise. The only specialist equipment required is a plasma cleaner to bond the PDMS device onto a substrate (typically a glass slide or another PDMS base). However, labs without access to this equipment can utilise a portable plasma 'torch' for bonding organ-on-chips (Corona SB, Elveflow Microfluidics). Alternatively, researchers can purchase ready-made devices that are immediately amenable to cell culture, such as the LiverChip® (CN Bio Innovations, United Kingdom) or Intestine Bio-Kit (Emulate Bio, USA). For further details on organ-on-chip fabrication methods, consult references[6,11,14].

Step 3: recreating the 'organ' in the organ-on-chip

Any given organ is functionally and architecturally complex. Therefore, we must be mindful that organ-on-chips serve to approximate these complexities by 'building the organ' using tissues or cells in culture. Nonetheless, with a fair amount of creativity and innovation, these approximations can recapitulate key functions and fundamental architecture of an organ unit. Recreating the functioning organ-on-chip relies on tissue culture work that is no different to traditional cell culture in flasks (Figure 4). Researchers

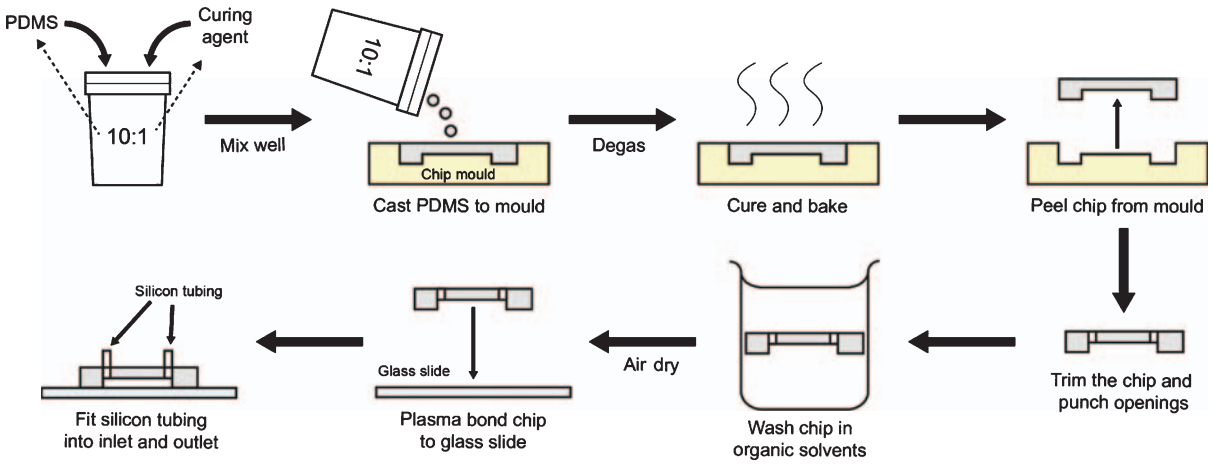

Figure 3. Workflow for organ-on-chip manufacture using PDMS. PDMS is a viscous fluid that solidifies when mixed at a recommended ratio of 10:1 with its curing agent. The mixture is then cast into the mould and baked at 95°C for curing. The PDMS chip is then peeled from the mould, trimmed and washed with organic solvent to remove residual uncured PDMS. The PDMS is then plasma bonded onto a glass slide, although other substrates can be used. Plasma activates the PDMS surface chemistry so that it forms irreversible chemical bonds when in contact with glass. Openings are punched into the PDMS and flexible silicon tubing fitted to create the device.

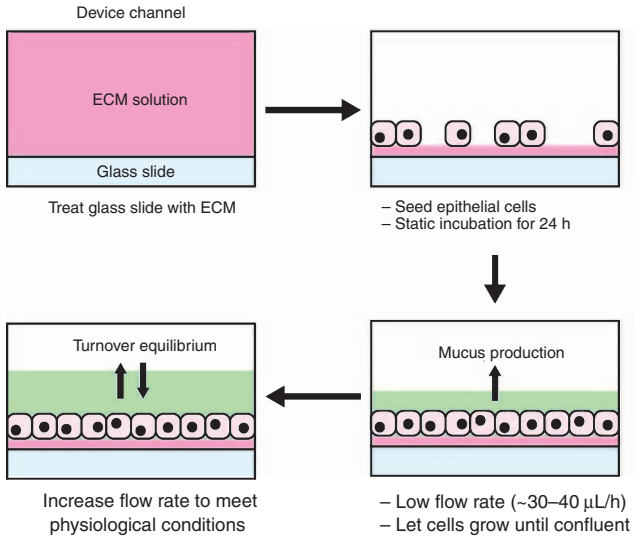

Figure 4. Workflow for reconstructing a mucus-secreting organ-on-chip. First, the device is first treated with biopolymers to provide cells with an extracellular matrix (ECM) to attach and grow within the channel[15]. The desired cell lines are propagated, harvested using standard trypsinisation and carefully seeded into the device using a micropipette. Cells are then incubated under static cell culture conditions to allow cell attachment to the ECM-treated substrate. Subsequently, the attached cells are perfused with tissue culture media to drive cell growth and differentiation within the organ-on-chip. In this example, a mucus-secreting gut epithelial cell line is used to recreate a gut-on-chip device.

need only to scale their techniques to efficiently handle tissue culture at the microfluid-level – a simple act of replacing serological pipettes with micropipettes.

Difficulties in transitioning culture cells from flasks into the organ-on-chip are often encountered, but can be overcome with a few simple solutions. Toxicity from uncured PDMS in the devices can potentially cause cell death, but is easily eliminated through organic solvent washes[16]. Determining the optimal cell seeding density will vary depending on the device and cell line used and often requires troubleshooting. Cell layer maintenance within the device requires a continual flow rate that does not impose excessive shear stress to the cells. Again, this will depend on the cell line used as some cell lines, such as endothelial lines, are more robust in withstanding high shear stress[17]. Consulting publications that have used similar cell lines and devices will provide a ballpark figure to start troubleshooting.

Step 4: operating the organ-on-chip

As outlined in step 3, cell growth and maintenance within the organ-on-chip is dependent on constant perfusion with culture media. Syringe pumps and pressure-driven systems are two widely adopted approaches to perfuse organ-on-chip devices, each with their advantages and limitations. Setting up syringe pumps is simpler and requires less tubing, but has limited flow control and sample inoculation options. Conversely, pressure-driven systems are computerised setups made up of multiple components to regulate air pressure that will drive fluid flow from a media reservoir into the device. Connecting these components requires various

adaptors and considerable tubing length, but offer increased flexibility for device control and inoculation. Furthermore, the computer interface in these systems offers fast response times and can incorporate flow sensor feedback loops that provide superior fluid flow stability compared to syringe pumps[18].

Moving forward: phage research in organ-on-chips

Traditionally, investigations of phage-bacteria interactions have been confined to *in vitro* broth culture. While these studies have proven instrumental for our understanding of phage biology, they neglect the complex environment and interactions seen *in vivo*. Recently, animal models have demonstrated the surprising diversity and stability of the phageome[19], and tissue culture-based *in vitro* studies have shown surprising interactions between phage and eukaryotic cells and tissues[4,20–22]. Organ-on-chip systems offer a unique way to study phage interactions within life-like systems that are cheap, accessible, and experimentally amenable.

Phage therapy approaches utilising organ-on-chip

Phages are known for their antimicrobial properties and are currently being pursued as an alternative to antibiotics in treating bacterial infections. Today, animal models are still the 'bread-and-butter' for preclinical testing of therapeutics, including the therapeutic validation of phages. However, animal models are costly, labour-intensive, and ethically questionable[9]. There are further concerns regarding the suitability of animal infection models to recapitulate human pathological conditions. Organ-on-chip models provide a middle ground between traditional static cell cultures and animal models for preclinical testing. A recent example was the use of a gut-on-chip to reproduce Coxsackie virus infection of a highly differentiated human villus intestinal epithelium, which reproduced cytopathic effects[3]. The use of organ-on-chip devices for phage therapy approaches offers large potentials, including the validation of antimicrobial capacity within an organ of interest, pharmacokinetic and pharmacodynamics studies, and tracking the emergence of phage resistance.

Gut-on-chip: moving gut phageome and microbiome studies from faeces to mucus

The human gut is home to a diverse repertoire of microbial species. This gut microbiome is comprised of trillions of microbial cells that influence our health, well-being and even psychological behaviour[23]. Numerically, the gut viruses, of which phages account for ~90%, are as abundant, if not more, than their microbial counterparts[24]. However, very little is known regarding the nature of phage-bacteria interactions within the gut. This is primarily due to the difficulty in studying and sampling the gut environment directly. Faecal samples are often used as a proxy to direct sampling, yet the faecal microbial communities differ significantly from intestinal mucosa[25]. Gut-on-chip devices address these limitations by providing a life-like environment for phage-bacteria experimental studies (Figure 4). This relatively simple set-up mimics essential aspects of the *in vivo* gut, namely the mucus layer, luminal flow, and spatial elements of the cell layer. Using gut-on-chip devices, it was demonstrated that phages were able to adhere to gut-produced mucus layer and as a result, exhibit enhanced antimicrobial activity within the mucus layer, providing a layer of non-host-derived immunity[4,20]. A microbiome gut-on-chip approach demonstrated the recapitulation of pathogenic microbially induced inflammation and the correction of these effects through probiotic and antibiotic therapies[26]. Finally, recent cell culture studies demonstrated that phages targeting the gut pathogen *Clostridium difficile* had increased antimicrobial affects when in co-culture with human gut cell lines[22]. These studies illustrate the potential of phage and microbiology studies within organ-on-chip devices.

Phage-bacteria ecology and evolution using organ-on-chip

To date, most evolutionary and ecological hypotheses attempting to explain phage-bacteria diversity in nature are confined to test-tube experiments and mathematical models. However, these are limited by the complexity of the experimental environment and assumptions of the models tested. Comparatively, the organ-on-chip approach allows for experimental investigations of these hypotheses under life-like conditions, adding increased complexity and biological relevance. Building off recent organ-on-chip microbiome devices[4,26], researchers are now able to study emergent microbial properties, such as co-evolutionary phage-host dynamics, experimental evolution of microbial communities, and investigations of gut phage-bacteria ecology. These devices are further amenable to the introduction of genetically modified phages and bacteria, including the insertion of fluorescence markers for real-time visualisation[27] or antibiotic or CRISPR locus for quantification of target populations[28,29]. The collective evolutionary and ecological results obtained may validate models and further explain gut microbiome diversity.

Conclusion

Phages have been at the forefront of many biological advances. Today, not only are they impacting the medical field through therapeutic applications, but also continually fueling fundamental

research, such as evolutionary biology and ecology. However, experimental phage research has been mostly confined *in vitro* and *in silico*. To that, we propose organ-on-chips as an experimental approach to further propel phage and microbiology research. The amenability of organ-on-chips allows researchers to conduct various phage and microbiological studies within life-like conditions; without the cost associated with animal models. Despite requiring high interdisciplinary knowledge, the organ-on-chip remains accessible to non-engineers through collaborations or simpler alternatives in setting up the platform.

Conflicts of interest

The authors declare no conflicts of interest.

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Use of bacteriophage for discovery of therapeutically relevant antibodies against infectious diseases

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Scientists George P Smith and Gregory Winter were recently awarded half of the 2018 Nobel Prize for Chemistry for developing a technology to display exogenous peptides and proteins on the surface of bacteriophage. 'Phage display' has revolutionised the development of monoclonal antibodies, allowing fully human-derived antibodies to be isolated from large antibody libraries. It has been used for the discovery of many blockbuster drugs, including Humira (adalimumab), the highest selling drug yearly since 2012, with US$18.4b in sales globally in 2017[1]. Phage display can be used to isolate antibodies to almost any antigen for a wide range of applications including clinical use (for cancer, inflammatory conditions and infectious diseases), diagnostic use or as research tools. The technology is accessible to any laboratory equipped for molecular biology and bacteria culture.

Displaying exogenous peptides and proteins on bacteriophage

Phage display technology was first demonstrated by Smith in 1985, who showed that DNA encoding peptides could be inserted into the bacteriophage gene III resulting in the expression and display of the corresponding peptides on the surface of the virion as a fusion to the coat protein pIII[2]. Winter then showed that this technology could be used to display antibody fragments on the surface of bacteriophage[3]. His group also showed that highly specific antibodies could be fished out of large libraries of antibody gene sequences cloned into phage expression vectors[4,5]. This now allowed the isolation of fully human antibodies, from cloned human antibody gene repertoires, reducing the impact of immunogenicity of mouse-derived therapeutic antibodies.

The bacteriophage biology that allows the display of peptides and proteins is well reviewed by Russel *et al.*[6]. The most commonly used phage display system uses phagemid vectors, where the antibody-pIII gene fusion is cloned into a bacterial expression vector containing a periplasmic leader sequence, an ampicillin resistance gene and an f1 viral origin of replication. When the phagemid is transformed into *Escherichia coli*, and grown in the presence of ampicillin and M13-derived filamentous helper phage (usually M13K07), the antibody-pIII fusion protein is expressed and incorporated into the newly synthesised phage particles, and the phagemid is replicated as single-stranded DNA and preferentially packaged into the particle (Figure 1). Phage particles are released into the culture media and are purified by precipitation with high salt and polyethylene glycol.

Phage display libraries and biopanning

Phage antibody libraries can either be 'naïve' or 'immunised'. Naïve libraries are usually human derived, and are created by collecting peripheral blood samples from a large group of healthy donors from a general population, with no bias towards any particular disease or condition. Naïve libraries can be used indefinitely to isolate antibodies to almost any target presented to the library. For this reason, naïve libraries are also termed 'single-pot' libraries since the same library can be used for any antigen[7]. Immunised libraries are focussed on the isolation of particular antibodies, with blood samples collected from individuals with a defined condition or from mice immunised with an antigen-of-interest[8]. Immunised libraries increase the likelihood of obtaining highly specific and high affinity antibodies, but also limits their use towards a single antigen.

The process of isolating specific antibodies from a phage antibody library is termed 'biopanning', and is summarised in Figure 2. Biopanning involves incubating the library of phage particles with immobilised antigen, washing away non-binding phage, and then eluting the bound phage using a buffer that breaks the antibody-antigen interaction. After enriching the library for binding phage, individual clones can be isolated, characterised and further developed as either laboratory tools, or as commercial diagnostic and therapeutic antibodies.

Therapeutic antibodies isolated by phage display

As of December, 2018, there were 82 antibodies approved in the US and/or EU for therapeutic use in humans, and 10 of these

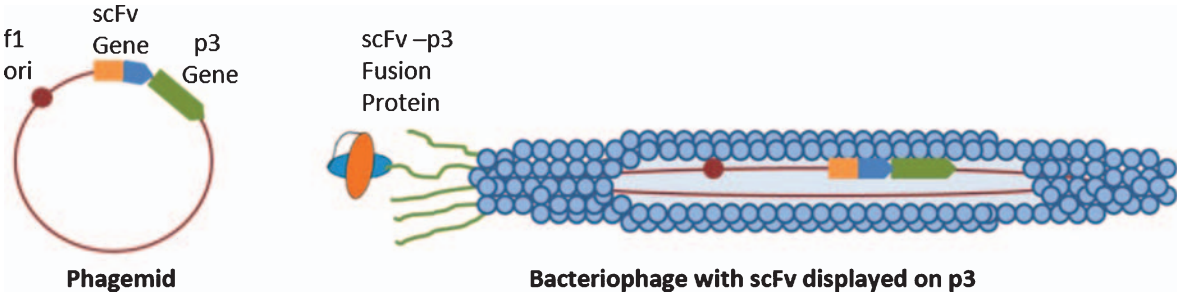

Figure 1. Left: A phagemid cloning vector containing an f1 origin of replication (f1 ori), and antibody variable region genes (Heavy chain (orange) and Light chain (blue)), assembled as a single chain variable fragment (scFv), cloned in frame with the gene for the bacteriophage p3 coat protein (green). Right: A bacteriophage particle containing a phagemid vector inside the particle, and the scFv antibody fragment displayed on its surface as a fusion to the p3 protein.

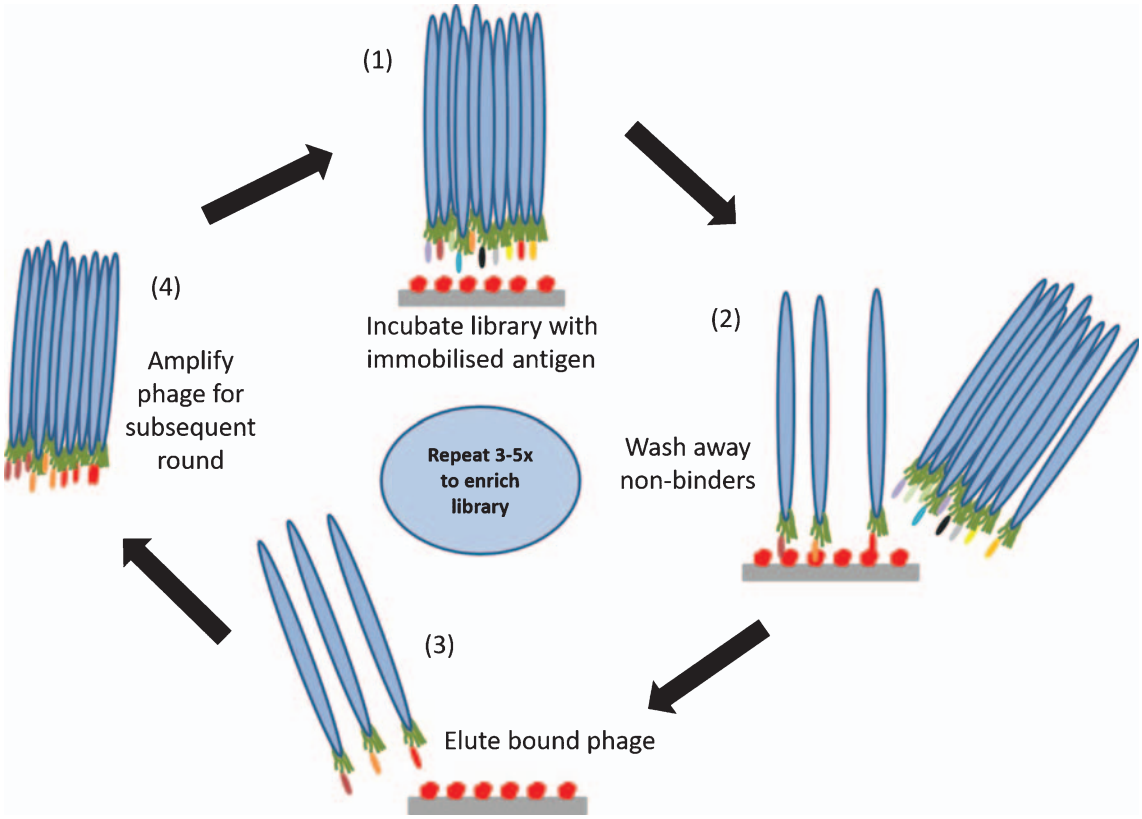

Figure 2. Summary of the biopanning process. The phage particles are depicted in blue with the scFv-p3 fusion protein on their tips. (1) The phage particles displaying a library of scFv is incubated with immobilised antigen (depicted in red), which could be purified proteins, or whole cells or viruses. (2) The surface is washed to remove any non-binding phage. (3) Bound phage are eluted using a low pH, high pH or high salt buffer. (4) The eluted phage are infected into *Escherichia coli* to amplify these phage, enriching the library for specific binders. This process is then repeated with the newly amplified, enriched pool 3–5 times with increasing stringency at step 2 to further enrich the library for strong binders.

were isolated using phage display[9–12] (Table 1). The majority of therapeutic antibodies target endogenous antigens such as proteins involved in the inflammatory response, or cell-surface or circulating proteins overexpressed in cancers.

Phage-derived antibodies against infectious agents

Therapeutic antibodies can also target infectious agents, including bacteria and viruses; examples include bezlotoxumab, which targets the B toxin of *Clostridium difficile*, obiltoxaximab and raxibacumab, which target the anthrax toxin, and palivizumab, which targets the F protein of respiratory syncytial virus. These are currently the only antibodies approved for therapy against infectious agents, and only raxibacumab was isolated using phage display. The others were isolated from mice using traditional hybridoma technology followed by humanisation, or using transgenic mice with humanised immune repertoires.

However, phage display, using immunised human antibody libraries created from individuals who have survived viral infections or from vaccinated individuals, offers a unique advantage for the isolation of neutralising antibodies to infectious agents. Antibodies have been isolated using such techniques from several viruses including Enterovirus 71[14], Ebola virus[15], HIV[16], West Nile Virus[17]

Table 1. FDA approved therapeutic antibodies isolated using phage display technology. Information was obtained from the ImMunoGeneTics antibody database (IMGT/mAb-DB)[11,13], and the numbers following each drug name indicate the IMGT database entry number.

Non-proprietary name	Trade name	Library type	Target	Indication	Year approved (FDA)
Adalimumab (IMGT-165)	Humira	Human naïve	TNF-α	Immune/ inflammatory diseases	2002
Ranibizumab (IMGT-84)	Lucentis	Mutagenic library of bevacizumab	VEGF-A	Immune/ inflammatory diseases	2006
Belimumab (IMGT-266)	Benlysta	Human naïve	B-lymphocyte stimulator	Immune/ inflammatory diseases	2011
Raxibacumab (IMGT-260)	ABthrax	Human naïve	Anthrax protective antigen of *Bacillus anthracis*	Infectious disease	2012
Ramucirumab (IMGT-295)	Cyramza	Human naïve	VEGFR-2	Oncology	2014
Necitumumab (IMGT-294)	Portrazza	Human naïve	EGFR	Oncology	2015
Ixekizumab (IMGT-380)	Taltz	Mouse immunised	IL-17A	Immune/ inflammatory diseases	2016
Atezolizumab (IMGT-526)	Tecentriq	Human naïve	PD-L1	Oncology	2016
Avelumab (IMGT-512)	Bavencio	Human naïve	PD-L1	Oncology	2017
Moxetumomab pasudotox (IMGT-198)	Lumoxiti	Mutagenic library of mouse antibody	CD22	Oncology	2018

and Rabies virus[18]. Neutralising antibodies can also be isolated from naïve human libraries using phage display. m102.4 antibody neutralises Hendra and Nipah viruses, and was isolated by panning a naïve library against the G-protein of Hendra virus[19]. This antibody has recently completed Phase I clinical trials in Australia[20] and has been used as passive immunotherapy in several individuals exposed to Hendra virus[21].

Biopanning strategies for isolation of antibodies to microbial targets requires a source of antigen for incubation with the phage library. The antigen can be a highly purified preparation of the target, for example viral proteins[22–24]or purified bacterial toxins[25,26], or crude preparations such as whole bacterial cells[27,28] or virus particles[29,30].

Advantages of phage display

Phage display offers several advantages over mouse immunisation strategies for antibody discovery, especially for targets that are either toxic or non-immunogenic in a mouse host, or where precision over epitope targeting is required[31]. Guidance towards particular epitopes can be incorporated into the biopanning strategy, by competing with a ligand, or alternating between mouse and human equivalent antigens, or depleting the library to binders that are cross-reactive to similar antigens. For example, antibodies specific for each of the four serotypes of Dengue virus (DENV) NS1 were isolated from a human naïve phage library[32]. Serotype specificity was achieved by first exposing the library to the other three DENV NS1 serotypes to deplete cross-reactive binders. Such antibodies may be useful in serotyping assays.

Phage display is a simple but powerful tool for antibody discovery, either for therapeutic use or for research tools. It is accessible to any laboratory equipped for standard culturing and molecular biology. Libraries can be created in-house, obtained commercially (Source Bioscience, Creative Biolabs) or shared from other researchers through material transfer agreements. Within Australia, the National Biologics Facility (NBF) at the University of Queensland offers phage display services and access to their naïve human library, and

has experience in isolating antibodies against infectious targets including Dengue virus[32] and the malaria parasite[33]. Isolation of viral neutralising antibodies using phage display of libraries generated from immunised or recovered patients is an emerging field in infectious disease therapy.

Conflicts of interest

Martina Jones is Operations Manager of the Queensland node of the National Biologics Facility, which offers phage display services to industry and academic groups.

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Biography

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Bacteriophages as biocontrol agents in aquaculture

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Aquaculture production (inland and marine) has been increasing globally reaching 80.1 million metric tons in 2016. Simultaneously the utilisation of fish food per capita has also been risen reaching 20.0 kg per year in 2016. However, the growing industry also experiences problems including diseases caused by viruses, bacteria, fungi, protozoans, helminths and parasitic crustaceans on valuable seafood products resulting in economic losses. Antimicrobial agents and chemical control strategies used to control such diseases are creating environmentally detrimental effects as well as encouraging development and dissemination of antibiotic resistant bacteria. Vaccine developments are costly and lengthy with application difficulties in farm settings. Accordingly, alternative therapies for controlling bacterial pathogens in aquaculture are gaining importance. One such measure is to use bacteriophages that are specific to disease causing bacteria.

Vibrio species are the main pathogens responsible for disease outbreaks which can result in 98.5–100% of mortality of the host animal within 72–96 h causing huge economic losses to hatcheries[1]. Examples include *V. tubiashii* infections that caused mortalities of oyster larvae in North America from 2006 to 2008 resulting in a decline of 59% in production at that time[2]. Moreover, antibiotic resistant species of *Vibrio* have also been on the increase. Mass mortality in the larvae of black tiger shrimp (*Penaeus monodon*) was reported to be caused by multi-drug resistant *V. harveyi* strains with resistance to cotrimoxazole, chloramphenicol, erythromycin and streptomycin[3]. Resistance of 15 *V. alginolyticus* isolates from oysters farmed in Korea against 16 different antibiotics including ampicillin, vancomycin and erythromycin has also been reported[4]. *Vibrio* spp. isolated from fish pond facilities in Nigeria were also reported to be resistant to tetracyclineoxazole (100%), oxytetracycline (99.4%) and chloramphenicol (73.1%)[5].

Aeromonas has been another pathogenic genus causing significant economic losses for aquaculture operations. Antibiotics again are extensively used to control diseases caused by the pathogenic species of this genus: examples include amoxicillin, ampicillin, cephamycin, cotrim and kanamycin[6,7]. However, according to the results from an antimicrobial susceptibility survey taken between 2013 and 2014, the sensitivity of these pathogens against the above-listed antibiotics decreased over this time thus devaluing the efficiency of antibiotic treatment. Highly virulent and antibiotic resistant strains to co-trimoxazole, tetracycline, florfenicol, ampicillin, trimethoprim/sulfamethoxazole, nalidixic acid, chloramphenicol, and nitrofurantoin were also reported[7–9]. Strains with complete resistance to methicillin, rifampicin, bacitracin and novobiocin were also reported for the same pathogen isolated from fish and prawns in South India[9].

Fish *nocardiosis* caused by *Nocardia* species in particular by *N. seriolae* is also on the increase in the South East Asia Pacific region. Erythromycin, oxolinic acid and fosfomycin resistant strains of the pathogen have also been reported[10].

While control of bacterial diseases has been attempted via different strategies during farming, after harvest unhygienic practices also constitute serious public health risk issues. Cross-contamination with pathogenic bacteria (e.g. *Escherichia coli*, *Campylobacter* and *Salmonella* spp) is one of the main causes of food poisoning after harvest. These pathogens can easily be spread to ready-to-eat foods, such as raw oysters and salads, through handling and

contaminated equipment or surfaces. In particular, during shucking of oysters, a significant risk of cross contamination can occur due to poor hygiene leading to gastrointestinal infections. The costs of foodborne diseases to the industry can be significantly high: e.g. US$10–83 billion in USA[11] and >AU$1.2 billion annually in Australia[12].

To reduce antibiotic use in the control of the above-mentioned pathogens in aquaculture farms alternative measures, in particular, those of biological origin, are being sought by the industry. One such measure is the use of bacteriophages that are specific to the disease-causing bacteria (Table 1). Phage therapy so far has displayed encouraging results in aquaculture settings via the use of diverse types of administrations: (1) direct application of phage suspensions in water; (2) oral administration of phages mixing with food; and (3) injections[13,14] (Table 2).

At the University of the Sunshine Coast (USC) in Queensland, Australian research in this field has also been carried out over the past 10 years and specific examples are listed below:

(1) Research study jointly conducted with the USC and the Research Institute for Marine Fisheries, Hai Phong and the Research Institute for Aquaculture No. 2, Ho Chi Minh, Vietnam, Le *et al*.[19] was able to reduce the incidence of disease due to *Aeromonas hydrophila* that causes Motile Aeromonas Septicemia (MAS) in Striped Catfish (*Pangasianodon hypophthalmus*). It is one of the most important farmed fish species in the South East Asia Pacific region including Vietnam, Thailand, Cambodia, Laos and, more recently, the Philippines and Indonesia[21]. In 2015, Vietnam supplied 90% of catfish production with a value of US$1.1–1.7 billion; however, an increase in Motile *Aeromonas* Septicemia cases and the detection of antibiotic resistant species of the pathogen has been threatening the productivity of the industry. Thus, the development of world first bacteriophage treatment against *Aeromonas hydrophila* with successful field trials conducted in Vietnam[19] now offers an alternative disease control strategy for the farmers.

(2) One of the main sources of *Vibrio* infections in aquaculture is the use of microalgae infested by the pathogen as feed in the aquaculture tanks. Bacteriophages were again successfully used to eliminate *Vibrio* infestations on microalgae used as a food source for oyster larvae in oyster hatcheries at the USC in a study jointly conducted with the Port Stephens Fisheries Institute in NSW, Australia. The morphology of one of these phages is illustrated in Figure 1*a*.

(3) Two key vectors for potential *Vibrio* spp. contamination in the hatchery include broodstock and seawater[22]. Bacteriophages were again successfully used to treat *Vibrio* infections in Sydney rock oyster larvae (*Saccostrea glomerata*) and this improved oyster survival rate in the USC in a study again jointly conducted with the Port Stephens Fisheries Institute in NSW, Australia. The morphology of one of these phages is illustrated in Figure 1*b*.

(4) Human pathogenic bacteria can contaminate sea-food because of unhygienic handling practices leading to foodborne diseases. This is a particular problem for oysters which are often eaten raw or only lightly cooked which might not remove human pathogens from the product[23]. Again, at the USC, Le *et al*.[20] successfully isolated five different *E. coli* phages and a *Salmonella* phage and treatments of shucked oysters with these phages resulted in significant decrease in the numbers of

Table 1. Possible advantages and disadvantage of biocontrol measures to aquatic bacterial disease[A].

Advantages	Disadvantages
Abundance in nature, including lytic and lysogenic bacteriophages	Only strong lytic bacteriophages are needed for phage therapy
Treatment does not require repeated administration	Difficult to extrapolate from *in vitro* treatment to *in vivo* expectation
Narrow host range can provide an effective treatment to targeted bacteria, without any effect on other bacteria	Need to identify and isolate the bacterium causing the infection/disease
Rapid process to isolate and select new lytic bacteriophages	Need expertise and experimental setting up and for careful screening to determine the activity spectrum of phages
Administration though feeding, injection and immersion	There might be practical difficulties e.g. injecting large numbers of aquaculture animals
High specificity of killing of pathogens, including antibiotic-resistant bacteria	Phage resistance can be developed by bacteria
Phage resistant colonies might be not pathogenic	Newly isolated phages are required for phage resistant bacteria
No side-effect to microbiota and environment during or after phage application	Phages could transfer virulence factors and other genes coding for undesired traits
Phage cocktails can reduce the phage-resistant-bacteria	All infecting bacteria must be exactly recognised that might have time constrains
Phage therapy might be less expensive than that of antibiotics	More studies in phage therapy might cause additional costs

[A]Adapted and modified from Oliveira *et al*.[13].

Table 2. Examples of successful application of bacteriophage treatments to control fish and shellfish diseases of bacterial origin.

Aquaculture food type	Etiologic agent	Bacteriophage	Source of isolation	Dose and application method used	Results	Reference
Adult oysters	*V. parahaemolyticus*	Phage VPp1	Sewage samples	Oysters were infected with 10^5, 10^6, and 10^7 CFU/mL of *V. parahaemolyticus* in the seawater and treated with three different multiplicity of infection (MOI)[A] values: 0.1, 1 and 10	Depuration at 16°C with 0.1 MOI phage treatment was the best condition for reducing *V. parahaemolyticus* in oysters, which decreased by 2.35–2.76 log CFU/g within 36 h	15
Shrimp larvae (*Litopenaeus vannamei*)	*V. parahaemolyticus*	A3S and Vpms1	A3S and Vpms1 were isolated from shrimp cultures and clams, respectively	MOI values: 0.1, 1, and 10	Phages effectively reduced the mortality rates of larvae caused by *V. parahaemolyticus*, especially when applied at the early larval stage (at 6 h post-infection)	16
Live prey (*Artemia salina*)	*V. alginolyticus*	φSt2 and φGrn1	Water samples from two locations of the north coastline of Crete, Greece	*In vivo* administration of the phage cocktail, φSt2 and φGrn1, at MOI = 100 directly on live prey *A. salina* cultures	93% decrease of presumptive *Vibrio* concentration after 4 h of treatment	17
Shrimp (*Penaeus monodon*)	*V. harveyi*	VHM1 (*Myoviridae*), VHM2 (*Myoviridae*) and VHS1 (*Siphoviridae*)	Water and sediments in Palk Strait, south east coast of India	Post larval stages of shrimp were treated with the test bacterium (10^5 cells/mL) first in laboratory trials followed by single phage treatment (about 10^9 PFU/mL) and phage cocktail treatment (about 10^9 PFU/mL)	Larval survival was 60–88.3% after 96 h in the presence of phage treatment, compared to 26.6–35% larval survival in the control treatments without phage	18
Striped catfish (*Pangasianodon hypophthalmus*)	*Aeromonas hydrophila*	*A. hydrophila* φ-2 and *A. hydrophila* φ-5	River water	All of the fish used in the treatment group were infected intraperitoneally with *A. hydrophila* N17 (3.2×10^6 CFU/fish) and were then immediately injected with a phage cocktail (MOI 0.01, 1 and 100)	The survival rate of catfish with MOI 100 was 100%, compared to the 18.3% survival in the controls unchallenged with the phages	19
Fresh edible Pacific oysters (*Crassostrea gigas*)	*E. coli* strains (JM 109, ATCC 13706 and ATCC BAA 196) and *S. enterica* subsp. *enterica* (ATCC 13311)	Five *E. coli* bacteriophages, φ-Eco1, φ-Eco2, φ-Eco3, φ-Eco5, φ-Eco6 and one for *S. enterica* subsp. *enterica* (ATCC 13311) (φ-S1)	Sewage water at a local sewage treatment plant in the Sunshine Coast region of Queensland, Australia	Bacterial contamination on surface of oyster meat (10^5 CFU/g of oyster meat) was treated with phage cocktail (10^{12} PFU/g oyster meat), along with bacterial control and phage control	A significant reduction for *S. enterica* subsp. *enterica* (ATCC 13311) with the final counts on the agar plate being 1.4 log (CFU/g oyster meat), compared to a control count of 5.7 log (CFU/g oyster meat) was obtained at the end of experiment *E. coli* (ATCC BAA-196) final concentration also indicated a significant difference between treatment of 6.3 log (CFU/g oyster meat) and the control of 7.9 log (CFU/g oyster meat) at 50 h	20

[A]Multiplicity of infection (MOI) is the ratio of infectious agents (e.g. phage or virus) to infection targets (e.g. cell).

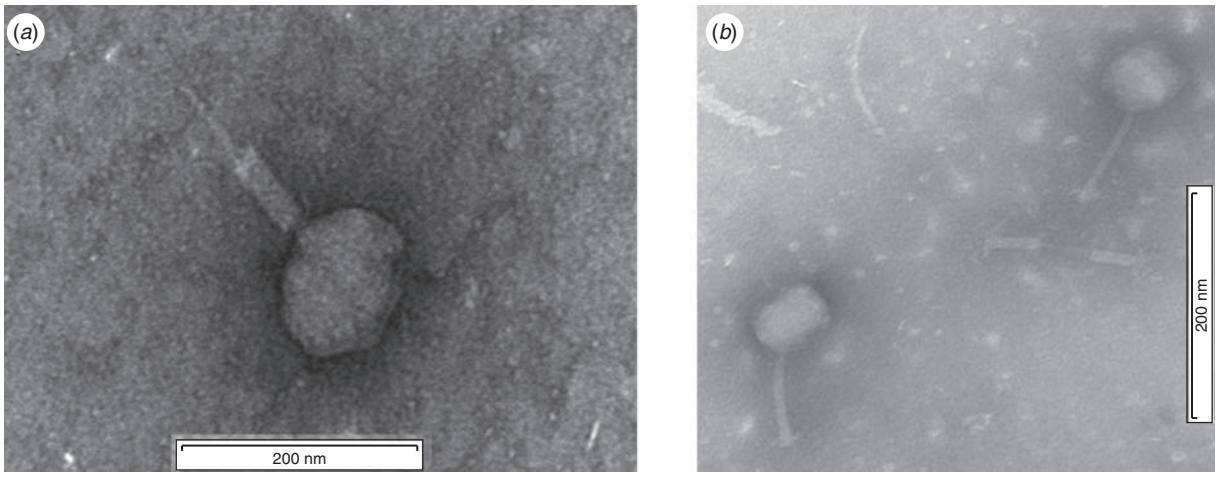

Figure 1. Transmission electron micrograph of *Vibrio alginolyticus* phages isolated in studies 2 (*a*) and 3 (*b*).

E. coli and *Salmonella*. Reduction in the numbers of extended spectrum beta-lactamase resistant *E. coli* strain (ATCC BAA 196) was also achieved[20].

(5) Moreover, off-flavor compound producing bacteria present in the sediments of unlined aquaculture tanks can result in the diffusion of earthy-musty compounds into fish flesh lowering the sale value of the product. Recently, in a joint study between the USC and the SeaFood Team of the Department of Agriculture and Fisheries in QLD, Jonns *et al*.[24] reported a decrease in odours caused by geosmin and 2-methyl-iso-borneol (2-imb) producing streptomycetes when they used streptophages in simulated aquaculture tank experiments in the laboratory. This method provides a safe alternative strategy to farmers whose business is detrimentally impacted by the odour producing bacteria e.g. the barramundi farmers.

Conclusions

The rising incidence of antibiotic resistance in bacteria and problems with antibiotic residues in aquatic environments and aquaculture products, highlight the need for, alternative therapies for control of pathogenic bacteria in aquaculture. Bacteriophage-mediated biocontrol can be one of these alternative methods[15–26]. The cases presented above demonstrate the potential of phage therapy in controlling diseases associated with aquaculture although further data is required for the acceptance and successful application of bacteriophages in aquaculture settings.

There are other factors to be considered before widespread application of bacteriophage therapy can occur such as existence of phage resistant bacteria. Examples include phage-resistant *Streptococcus iniae* causing beta-hemolytic streptococcicosis in Japanese flounder *Paralichthys olivaceus*[27].

Bacteriophages can also mediate toxicity such as the one encountered when *Penaeus monodon* gets infected with *V. harveyi*[28]. Accidental introduction of lysogenic phages was pointed out as an inherent risk for shrimp farmers[29]. *V. harveyi* Siphophage 1 (VHS1) was found to lose its ability to lyse cells but retained its ability to lysogenise after boiling for 10 min. Accordingly, cooking of crustaceans may not be sufficient for full inactivation of phages that might be present in the seafood thus resulting in lysogenic conversions[29].

In-depth understanding on the fascinating interactions between the host and bacteriophages will facilitate development of effective management systems including the use of several techniques in rotation including the bacteriophage therapy[13].

Conflicts of interest

The authors declare no conflicts of interest.

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Biographies

Son Tuan Le currently is a PhD student at the University of the Sunshine Coast (USC) as well as an environmental researcher at Research Institute for Marine Fisheries (Vietnam). He obtained his Bachelor of Environmental Science degree in 2009 from the Vietnam National University in Hanoi. He subsequently obtained his Master of Fisheries Sciences degree in 2012 at the Pukyong National University in the Republic of Korea. His MSc research project involved the application of bacteria for biodegradation of fish waste water in Korea. He then moved to Australia for his PhD studies at the University of the Sunshine Coast under the principal supervision of Dr İpek Kurtböke where he investigates the use of bacteriophages to control bacterial diseases in aquaculture. He is currently the recipient of the MOET-VIED/USC PhD scholarship.

Dr İpek Kurtböke has been working in the field of biodiscovery and has been an active member of the international actinomycete research community since 1982. She currently conducts research and teaches in the field of applied microbiology and biotechnology and is senior lecturer at the University of the Sunshine Coast (USC), Queensland. She has also been an active member of the World Federation of Culture Collections (WFCC) including serving as the Vice-President of the Federation (2010–2013) and currently is the President of the Federation (2017–2020).

Future prospects of structural studies to advance our understanding of phage biology




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Bacteriophages, being the most abundant biological entities on the Earth, play a major role in regulating populations of bacteria and thus influence the evolution and stability of ecosystems. Phage infections of pathogenic bacteria can both exacerbate and alleviate the severity of the disease. The structural characterisations of phage particles and individual proteins have enabled the understanding of many aspects of phage biology. Due to methodological limitations, most of the structures were determined from purified samples *in vitro*. However, studies performed outside the cellular context cannot capture the complex and dynamic interactions of the macromolecules that are required for their biological functions. Current developments in structural biology, in particular cryo-electron microscopy, allow *in situ* high-resolution studies of phage-infected cells. Here we discuss open questions in phage biology that could be addressed by structural biology techniques and their potential to enable the use of tailed phages in industrial applications and human therapy.

State-of-the-art structural biology methods in studies of phage replication

Bacteriophages are a diverse group of viruses that infect bacteria. Bacteriophages are research models for molecular biology and have the potential to be used in modern biotechnology and phage therapy. After ejecting their genome into a cell, bacteriophages can establish two types of infection. The lytic cycle leads to the production of virion progeny and cell death, whereas in lysogenic infection the phage genome integrates into the bacterial one and replicates when the bacterium divides. Knowledge of the high-resolution structures of phage particles and their assembly intermediates has played an important role in our understanding of phage attachment to receptors, genome ejection, virion assembly and genome packaging. Furthermore, the structures of non-structural proteins and their complexes have explained the mechanisms of the lytic-lysogeny switch, genome transcription and replication, and the degradation of the cell envelope. However, nearly all of the structural studies performed to date were limited to analyses of purified macromolecular samples *in vitro*. In contrast, phage macromolecules perform their functions *in vivo* by interacting with other phage or cellular components. Current technological developments in the cryo-preservation of cells and cryo-electron tomography (cryo-ET) have enabled structural studies of replicating phages in bacteria. These reports described the ultrastructure of bacteriophages penetrating the cell wall of Gram-negative and Gram-positive bacteria, the formation of a nucleus-like structure during phage replication, and changes in the structure of the cell wall before lysis[1–3].

*These authors contributed equally.

Structural virology beyond purified proteins and phage particles

In the single-particle cryo-electron microscopy (cryo-EM) approach, a sample is deposited on a grid in a thin layer of aqueous solution and rapidly plunged into liquid ethane[4]. This results in the formation of vitreous ice with a structure similar to that of liquid water. Rapid cooling is required to prevent the formation of crystalline ice, which may damage cellular structures. This limits the thickness of samples vitrified under ambient pressure to a few micrometres. Samples up to a few hundred micrometres thick can be cryo-preserved by high-pressure freezing. Individual molecules or macromolecular complexes embedded in vitreous ice are photographed using a transmission electron microscope. Information from many thousands of the projection images of the macromolecules is used to reconstruct their three-dimensional structures. The images are aligned and averaged to improve the signal-to-noise ratio of the reconstructed structure. Pleomorphic objects, such as cells or irregular virus particles can be studied by cryo-ET[5]. Cells have to be thinned to about 200 nm by cryo-sectioning or focused ion beam (FIB)-milling before imaging in a transmission electron microscope because of the limited penetration of electrons through biological samples (Figure 1). In cryo-ET, samples are imaged from different directions by tilting the stage of the microscope. The resulting tilt series of images is used to calculate the three-dimensional reconstruction of the object. The sensitivity of biological objects to an electron beam limits the overall dose that can be used to image one sample, resulting in a low signal to noise ratio in the reconstructed tomograms. However, sub-tomogram averaging can be used to resolve the structures of regular components of the tomograms with high contrast and resolution.

Currently, high-resolution structures can only be routinely determined for protein complexes or phage particles that can be prepared with high purity and at high concentration. These experimental constraints limit the knowledge that can be gained from the resulting structures, because the complexes may display different conformations *in vivo*. Determining the structures of macromolecular complexes *in situ* without the need to purify them from cells would avoid these experimental limitations. Structural analyses of macromolecular complexes *in situ* are becoming practical thanks to developments in: (1) sample preparation methods, including correlative light and electron microscopy (CLEM), focused ion beam milling, and localised mass spectrometry[6]; and (2) software for data processing including sub-tomogram classification and averaging[7]. CLEM is particularly useful in the combination with FIB-milling technique. Events of interest in the cell can be pre-selected by cryo-fluoresce microscopy and subsequently milled with high-precision to open a 'window' into the cell for transmission electron microscopy. The localised-mass spectroscopy utilises imaging of single particles from a cell extract. First, the cell lysate is chromatographically separated into fractions. Subsequently, the fractions are

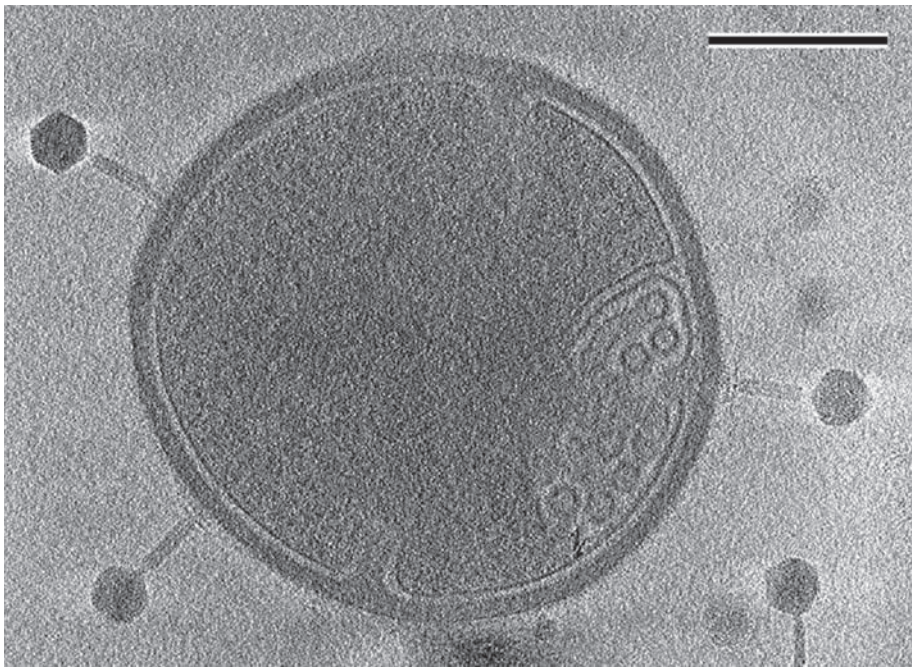

Figure 1. Cryo-electron tomography of *Staphylococcus aureus* cell infected by bacteriophage ϕ812 from the family *Myoviridae*, genus *Kayvirus*. Central section of *S. aureus* cell with resolved cell wall, cytoplasmic membrane, and vesicles formed inside the cell. Acquisition of the tomographic data was possible thanks to the use of focused ion beam milling to prepare an electron transparent lamella of *S. aureus* cell. Scale bar represents 200 nm.

characterised by mass spectrometry and macromolecules present in the sample are structurally analysed by transmission electron microscopy. The new advancements in software for classification of particles allow classification of particles in a sample based on their structures. This can be imagined as an *in silico* 'purification' of the macromolecular complexes.

Open questions in phage biology that may be addressed by structural studies

Mechanism of phage genome delivery

Tailed phages eject their genomes into bacterial cells, however, several aspects of this process are not well understood (Figure 2), including: (1) How is the phage genome ejection triggered? (2) How is the genome transported across bacterial membrane(s)? (3) What is the mechanism for the completion of phage genome ejection after the pressures inside the phage head and cell equalise? (4) How is the transcription machinery recruited to the phage genome? These questions may be addressed by cryo-EM observations of interactions of phages with liposomes[8], nevertheless, more biologically relevant answers will be obtained by time-resolved cryo-ET studies of phage genome ejection into mini-cells or focused ion beam milling-prepared sections of bacteria[9,10]. Changes in the phage particle prior to genome ejection, formation of channels in the membrane, possible genome-uptake machinery or phage genome localisation in the cell may be characterised by such studies. Single particle cryo-EM and X-ray crystallography can be used to determine the structures of the complexes of phage receptor-binding proteins with the receptors. Such knowledge may make it possible to design a group of genetically modified phages with a receptor range so wide that bacteria would be incapable of becoming resistant to the phage infection. Phage receptor-binding proteins themselves may be used as tools for the rapid detection and identification of pathogenic bacteria in environmental samples.

Bacterial resistance to phage infection

Mutations enabling bacteria to avoid phage attachment or block genome ejection can have secondary effects on the cellular phenotype. Phage receptors are often bacterial virulence factors or play roles in substances intake[11], and thus phages targeted to bind to specific cellular receptors could be used to shift bacterial population towards lesser virulence[12].

Bacterial anti-phage defense systems, such as restriction-modification, CRISPR/Cas, bacteriophage exclusion, or the defense island system associated with the restriction-modification system, degrade phage DNA during delivery[13–15] (Figure 2). However, many phages have acquired anti-defense proteins[16,17]. Structural understanding of the anti-phage defense complexes will enable the preparation of phages capable of protecting their DNA during delivery, which may be important for the development of phage therapy. Similarly, the systems by which bacteria abort late stages of phage infection are assumed to be widespread, but many of them have not been characterised in detail[18]. The CLEM and cellular cryo-ET could be used to visualise these processes *in vivo* and explain the functions of these complexes. These findings will enable the engineering or phages with exceptionally broad host-ranges.

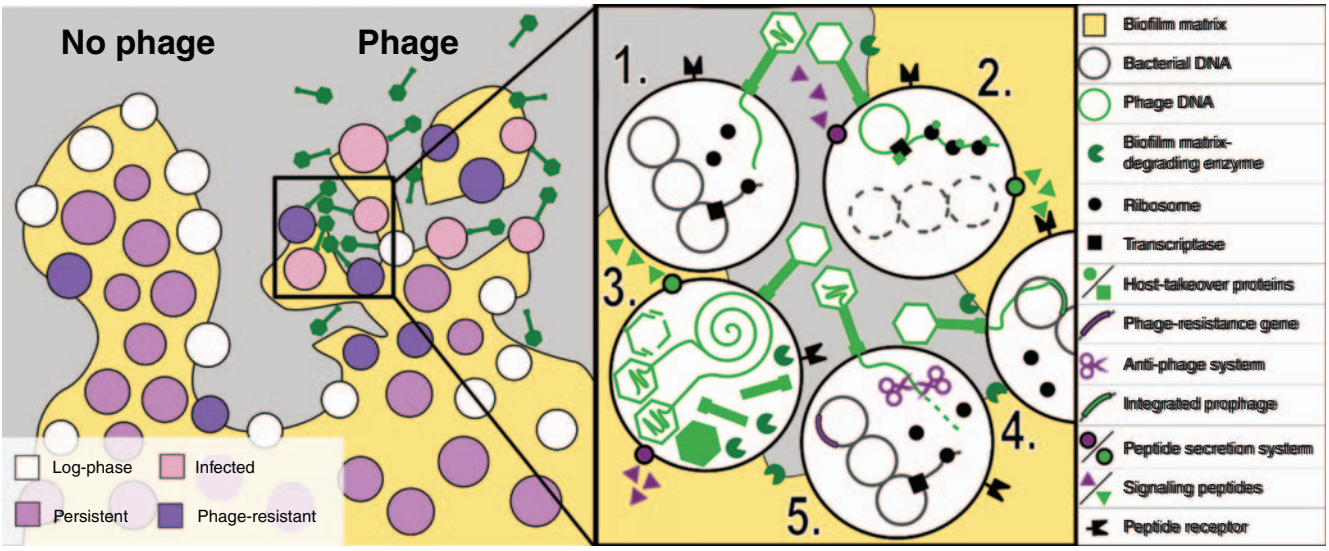

Figure 2. Open questions in our understanding of phage infection of bacterial biofilm that may be addressed with the use of structural biology methods. Cells in a biofilm are in different metabolic states. Phage infection may result in degradation and dispersion of biofilm due to a phage-driven expression of biofilm matrix-degrading enzymes. Inset: (1) What is the trigger of phage genome ejection? What is the mechanism for ejection of phage DNA after equalisation of pressures inside phage head and bacterial cytoplasm? (2) How is the host replication, transcription, and translation machinery hijacked for the expression of phage genes? (3) How do matrix-degrading enzymes enhance dispersion of phages through a biofilm? (4) What are the mechanistic details of macromolecular complexes enabling phage-phage communication in the regulation of lysis-lysogeny decisions? (5) What are the mechanisms of function of bacterial anti-phage systems?

Subverting cell resources for phage replication

Some myoviruses, such as T2 and T4 infecting Gram-negative bacteria and K and SPO1 of Gram-positive bacteria, degrade the host genome and block cell division in minutes after the initiation of genome ejection[19,20]. This mechanism enables them to complete their lytic cycle quickly and thus gain a reproductive advantage over slower replicating phages. The proteins encoded by the host takeover region of the phage genome, which is the first part of its DNA that enters the cell, enable the rapid shutdown of the host's transcription[21]. When expressed on their own, these proteins are toxic for the natural host of the phage but also for other bacterial species[20]. In contrast, some podoviruses such as ϕ29 do not inhibit cell growth[22]. Genomes of viruses from the family *Podoviridae* contain fewer than 20 early genes and during phage infection only affect the expression of a minor number of host genes[22]. Therefore, podoviruses are a suitable model system for studying the minimal set of phage products that are required to hijack the host resources for phage replication. The interactions of phage proteins with host complexes could be studied by the time-lapse cryo-EM of macromolecular complexes pre-sorted by mass spectrometry[23]. Identification of the host takeover protein-machinery may enable design of antibiotics inspired by phage proteins[24].

Phage spread through biofilm

Biofilm inactivation is a major healthcare and food-hygiene challenge. It has been shown that some phages can eliminate a biofilm thanks to their ability to (1) bind to and accumulate within the biofilm matrix; (2) infect dormant cells; (3) express phage biofilm de-polymerases or induce bacteria-encoded biofilm depolymerases[25]. Nevertheless, biofilm infection by some lytic phages can lead to accelerated biofilm growth with an increased concentration of extracellular DNA in the biofilm matrix[26].

The metabolic heterogeneity of cells within a biofilm presents a challenge for analysing the impact of the phage infection on the biofilm (Figure 2). Identifying the genetic markers of fast proliferating cells, anaerobically growing cells, starving cells, and persister cells will enable the differentiation of bacteria by fluorescence microscopy to study their unique interactions with phages by cryo-ET. However, some phages can form particles with different propensities to infect starved cells[27]. CLEM and cryo-ET studies of biofilm infection by bacteriophages will determine whether and how phage particles distinguish between metabolically distinct host cells. Mechanisms that allow phages to diffuse through the biofilm matrix are of interest because they may be used to enhance penetration of antibiotics into biofilms. Furthermore, phage-derived nano-vehicles may be used for the targeted delivery of drugs into biofilms.

Phage-phage communication in the regulation of lysis-lysogeny decisions

It is beneficial for phages to establish lysogeny in an environment with a shortage of non-infected host cells[28]. For the application of phage therapy, however, lysogeny is not desired because of the possible associated acquisition of bacterial virulence factors[29]. The communication among phage-infected cells and phages was proven in several cases, either in the most simple form of superinfection exclusion regulated by changes in the bacterial membrane potential[30], complex communication by the production of Arbitrium-like peptides[31], or through a host-produced quorum-sensing system[32]. Even lytic phages were shown to modify the speed of their reproduction cycle based on available nutrients and enter the 'hibernation' phase in starved cells[33]. X-ray crystallography and nuclear magnetic resonance spectroscopy can be used to determine the structural interactions between the complexes responsible for such communication. Understanding this communication may enable the use of temperate phages for phage therapy in bacterial pathogens for which there are no available strictly virulent phages. In lytic phages it can lead to design of small-molecule additives, ensuring the lytic cycle will be rapid and robust.

Conflicts of interest

The authors declare no conflicts of interest.

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Use of bacteriophages as biological control agents in horticulture



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Bacterial diseases in horticultural settings or infestation of fresh produce with human pathogenic bacteria can constitute a serious public health risk. To control horticultural bacterial diseases, chemical control strategies have traditionally been used, such as the application of bactericides and copper-based products, which resulted in development of resistance in bacteria against these agents. Moreover, the use of such chemical preventative measures on fresh produce can detrimentally affect human, animal and ecosystem health. Bacteriophages have been used to control pathogenic bacteria since the 1920s due to their specificity against host bacteria, as well as their ability to survive and infect their host without detrimental effects to the surrounding environments. As a result, their targeted host specific applications in horticultural settings can be of interest to growers as well as to the consumers. In this laboratory report, the efficacy of a bacteriophage cocktail when applied to fresh herbs inoculated with *Escherichia coli* was determined. Significant ($P \leq 0.001$) reductions in *E. coli* colony forming units were observed in phage treated herb samples compared to counts in the control. These findings suggest that bacteriophage present as an alternative biocontrol for *E. coli* in horticulture.

Xanthomonas campesteris pv *campesteris*, the cause of black rot in brassicas, was one of the first bacteria to be challenged by a phage battery in 1924[1,2], followed in 1925 by isolation of phage active against *Pectobacterium carotovarum* subsp *atrosepticum*[1,3] that resulted in the prevention of potato tuber rot. Field trials date back to the mid-1930s when corn seeds were treated with bacteriophages specific to *Pantoea stewartia* that resulted in significant reduction in Stewart's wilt disease incidence[1,4]. In the late 1960s a laboratory trial demonstrated that the use of bacteriophage significantly reduced bacterial spot infection in the leaves of peach seedlings caused by *Xanthomonas pruni*[1,5]. More recently, Kurtböke *et al*.[6] demonstrated effective elimination of human pathogenic *Enterobacteriaceae* species contaminating strawberries post-harvest using bacteriophage suspensions containing multiple polyvalent phages targeting the members of this bacterial family. In another study jointly conducted by Terragen Biotech Pty Ltd and the University of the Sunshine Coast (USC) in Queensland, Australia, Ashfield-Crook *et al*.[7] investigated the control of potato scab causing streptomycetes using streptophages. Again, another recent study conducted at the USC targeted the control of *E. coli* test strains using locally grown herbs. This laboratory report will present some of the preliminary findings of this study.

E. coli is a facultatively anaerobic bacterium that can survive outside of the host in fecal matter and soil[8]. Although most strains of *E. coli* are harmless, a few pathogenic strains such as serotype O157:H7 can cause serious infections in humans such as haemorrhagic enteritis[9], with some rare cases leading to bowel necrosis,

septicemia and haemolytic uraemic syndrome[10]. Many strains of *E. coli* can contaminate fresh produce including herbs[11], lettuce[12], spinach[10], vegetables[13,14], and herbs like coriander that has been reported to be contaminated by *E. coli* 0157:H7[15]. To control such pathogenic bacteria, antimicrobial treatments have traditionally been used[16]. However, due to the rise in antibiotic resistance among human pathogenic bacteria[17], alternative biocontrol agents and strategies are needed.

Bacteriophages can be effectively used as a control method on fresh produce contaminated with pathogenic bacteria including *E. coli*[6,18] and as their application is less destructive to the natural habitat they can also be used on edible food[16,19]. Examples include *Listeria monocytogenes* specific phage (P100) that has been rated as GRAS by the US FDA, the EU EFSA and Australian FSANZ, and commercially available as Listex™ to control this pathogen in RTE foods[20].

The objective of this study was to use a bacteriophage cocktail composed of eight different phages as a biocontrol agent against *E. coli* (JM109), used under laboratory settings to deliberately infect five different locally grown herb samples. Additionally, the effectiveness of the phage cocktail against the same *E. coli* under natural settings was tested using pot parsley plants.

Eight different bacteriophages were obtained from the Microbial Library of the USC and their characteristics were previously described[6]. Each phage sample was propagated on *E. coli* (JM109) (https://www.atcc.org/Products/All/69905.aspx) with a titer of $\sim 10^{10}$pfu/mL. A bacteriophage cocktail was then prepared using each individual phage sample in equal volumes and used to treat herbs contaminated with the test strain.

Herb samples; parsley, coriander, mint, Vietnamese mint and rosemary were obtained from a local supplier and were surface sterilised[6] to ensure removal of any microbial contaminants that might be originating from the environment. Each herb leaf was then inoculated with JM109 and left to stand for 10 minutes to allow absorption of the bacterium into the plant tissue. Serial dilutions of the infected leaf samples were performed and from selected dilutions inoculations were made onto Tryptic Soy Agar (TSA) (OXOID, Australia) in triplicate. The phage-treated group of leaf samples were submerged into the bacteriophage cocktail solution for 1 hour. The phage treated herb leaves were then subjected to 10-fold serial dilutions and plated out in the same way as the control samples.

In the second phase of the study, potted parsley plants were obtained from a local supplier and they were divided into four different treatment groups: (1) a control with neither *E. coli* nor phage cocktail; (2) a control treated with *E. coli* only; (3) a treatment group exposed to both *E. coli* and phage cocktail; and (4) a third control treated with phage cocktail only.

In contrast to the two different controls (one with no JM109 or phage cocktail exposure, the other one exposed to phage cocktail only), two of the potted parsley plants were deliberately infected with ~5 mL of JM109 by gently rubbing the strain onto the plant using sterile gloves. The two pots containing infected parsley samples were first incubated at room temperature for 10 min. Parsley samples from one of the JM109 treated pot plants were cut and soaked in sterile distilled water. The samples from the second pot were first exposed to the phage cocktail for 1 h and then cut and soaked in sterile distilled water. The first potted parsley plant served as a control without any JM109 or phage cocktail application. All parsley samples from all of the treatments were shaken on an orbital shaker for 15 min at 110 rpm in 37°C and subsequently subjected to serial dilutions. Aliquots (200 µL) from selected dilutions for all potted parsley samples were finally inoculated in triplicate onto both TSA plates for general bacterial counts and MacConkey (OXOID, Australia) for its selectivity toward *E. coli*. Results were analysed using Student's *t*-test[21].

Use of the bacteriophage cocktail reduced the JM109 colony counts on all of the tested herbs with a high degree of significance ($P \leq 0.001$), resulting in complete lysis. An example of full plate clearance is illustrated in Figure 1.

When aliquots were taken from potted parsley sample suspensions and inoculated onto either the TSA or the selective MacConkey agar plates, JM109 numbers were again found to be significantly reduced if the parsley samples were treated with the phage cocktail compared to the untreated control treatments ($P \leq 0.001$) (Figure 2). The phage cocktail did not demonstrate lytic activity against the resident microflora present on the parsley prior to inoculation with JM109 (Figure 2, bottom plates).

The bacteriophage cocktail successfully reduced the numbers of JM109 on each different surface-sterilised herb indicating that surface structure or chemical compositions of the herb plant did not display significantly different interference with the phage activity. Moreover, bacteriophage activity was also persistent on non-surface-sterilised potted parsley samples when they were deliberately contaminated with the *E. coli*. Since the JM109 is a highly engineered strain of *E. coli,* the technique was also tested using other *E. coli* species (ATCC 25922 and ATCC BAA-196: ESBL +ve, as well as using local isolates listed in Kurtböke *et al*.[6] using only parsley as the test herb. Again, significant reduction in the numbers of the tested different *E. coli* strains were achieved

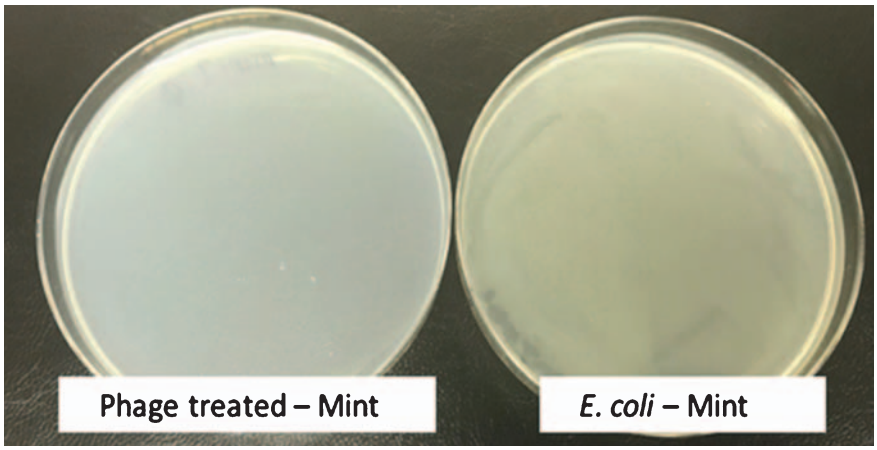

Figure 1. Phage treatment of *E. coli* (JM109) inoculated on mint resulted in the absence of growth following incubation on TSA (left). Plates without the phage treatment resulted in confluent growth of *E. coli* (right).

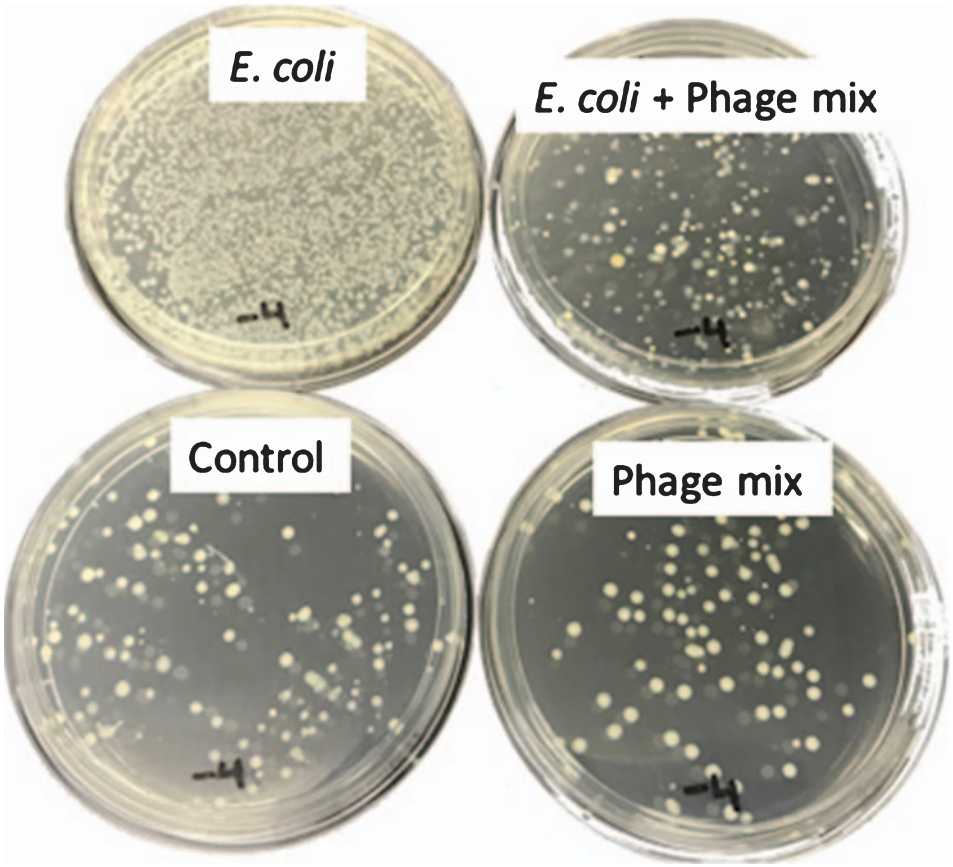

Figure 2. A reduction of the colony forming units of *E. coli* inoculated parsley samples treated with and without phage was observed (top plates). The phage cocktail had no lytic activity against the parsley's resident microflora (bottom plates).

($P \leq 0.001$) (İ Kurtböke, 2015, unpublished data). All these findings were in line with other studies where successful bacteriophage applications were reported[6,18] and suggest that bacteriophage biocontrol strategies might be an alternative to chemical controls used in horticultural settings. However, as stated by Jones *et al.*[1], a number of factors should be considered during phyllospheric applications of the phages such as establishment of high-density phage populations in close proximity to the pathogen targeted for control at critical times in its disease cycle. Environmental factors may impact phage survival and persistence; such as inactivation by UV[22] that would impact phage survival and persistence. Accordingly, the design of phage protective delivery methods is of importance as well as careful monitoring of the phages during field use to minimise development of resistance by the targeted host bacteria. Recently, Ashfield-Crook *et al.*[7] also demonstrated that polyvalent phages might also have unintended consequences in field applications by simultaneously removing beneficial microflora and resulting in increased risk of secondary infections. Although bacteriophages have significant potential to be utilised as biocontrol agents in agricultural and horticultural settings, the generation

of further data and careful observations in the field have critical importance for their acceptance as reliable disease control agents.

Conflicts of interest

The authors declare no conflicts of interest.

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Biographies

Rhianna O'Regan is a graduate of the University of the Sunshine Coast (USC). She completed a *Special Research Project* course under the supervision of Dr İpek Kurtböke working on the bacteriophage control of herb infesting *E. coli* pathogen. Following her graduation, she worked at Q-Pharm in Brisbane QLD, as a Clinical Laboratory Officer for 2 years and currently is returning to the USC to conduct her MSc studies under the supervision of Dr İpek Kurtböke on fungal diseases associated with corals to be investigated jointly with the co-supervision of Dr David Bourne at JCU.

Annaleise Wilson is also a graduate of the USC. She completed a *Special Research Project* course under the supervision of Dr İpek Kurtböke working on the bacteriophage control of milk contaminating *E. coli* pathogen. Following her graduation, she continued with her Honours studies under the supervision of Dr İpek Kurtböke in a project linked with the CSIRO *Food Safety and Stability* research group led by Dr Narelle Fegan in Melbourne and recently graduated with first class Honours. She is now a PhD student at the UQ studying molecular pathogenesis of food borne pathogens under the supervision of Prof Mark Turner.

Dr Ipek Kurtböke has been working in the field of biodiscovery and has been an active member of the international actinomycete research community since 1982. She currently conducts research and teaches in the field of applied microbiology and biotechnology and is senior lecturer at the USC. She has also been an active member of the World Federation of Culture Collections (WFCC) including serving as the Vice-President of the Federation (2010–2013) and is currently the President of the Federation (2017–2020).

Introduction of the Bacteriophage Biology & Therapeutics SIG

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Bacterial viruses, more commonly known as bacteriophages, are by far the most numerous and diverse virus type. More than 100 years since their discovery, the contribution of bacteriophages to fundamental biology, biotechnology and human health continues unabated. Access to new technologies, the growing antibiotic resistance crisis and a surge of new researchers entering the field have all contributed to a phage biology renaissance. Here in Australia, phages are being studied in the context of the microbiome, agriculture and aquaculture, synthetic biology approaches are being used to engineer phages, innovative phage delivery approaches are being developed, and there is an increasing number of translational phage therapy studies being conducted, including clinical trials and compassionate usage cases. As homage, this issue of *Microbiology Australia* is devoted to bacteriophages.

I would like to take this opportunity to promote the Bacteriophage Biology & Therapeutics Special Interest Group (SIG) as part of the Australian Society for Microbiology (ASM) framework. Our goal as a bacteriophage SIG is to support phage research within Australia through associations with the ASM; however, our hope is that the SIG can go well beyond this and shape Australian phage research in a number of ways.

Phage research can be challenging, especially for newcomers to the field. As such, a major goal for the SIG is to develop a collaborative network of phage biologists willing to share knowledge, methods, resources and support for phage researchers within Australia, with a particular focus on providing guidance and support for early career researchers. Having access to such a collaborative network can lead to new research directions, seminar and conference invitations, joint funding opportunities, training programs and job opportunities. In my opinion, this network is the greatest potential of the SIG.

The Bacteriophage Biology & Therapeutics SIG will promote Australian phage research on both a national and international scale. To date the SIG has helped organise phage symposium sessions at 2018 Molecular Microbiology Meeting (MMM), the ASM 2018 Meeting and the upcoming ASM 2019 Meeting. Further, our members regularly attend and present their research at leading international phage conferences, including Viruses of Microbes and the Evergreen Phage Meeting, often communicating emerging research and trends presented at these conferences to our SIG members.

The SIG also looks to have an influential role in shaping phage therapy within Australia. Connections with numerous stakeholders, including academic researchers, clinicians, biotechnology companies, entrepreneurs, government officials, and the general public are all incredibly important for the translation of phage therapy. Over the coming years the SIG will facilitate collaborations and meetings with interested parties, communicating our involvement in these processes, in order to move translational phage therapy within Australia forward.

In order to accomplish these goals, we have set up a blog site – https://bacteriophagesig.blogspot.com/ – to better communicate with SIG members and other interested parties. Every three months this site will host a short blog post on phage research and will further update our members on SIG activities, conferences, job postings and other related information.

Finally, I would like to encourage anyone reading this issue who has an interest in phages and would like to participate in, or be updated by the Bacteriophage Biology & Therapeutics SIG, to please reach out to myself or any other SIG member. Now is the time to adsorb and propagate the phage.

Book review

The Squid, The Vibrio & The Moon

Ailsa Wild, Aviva Reed, Briony Barr, Gregory Crocetti and Linda Blackall

CSIRO Publishing, 2019

A Hawaiian bobtail squid, *Vibrio fischeri*, and the moon: three seemingly unrelated characters with a mysterious evolved relationship that is revealed in this engaging, delightful and colourful children's story. It's a dramatic tale of squid development and the uptake of symbiotic *Vibrio* cells, which provide bioluminescent camouflage against the moon as the squid hunts at night, protecting the squid from dangerous predators such as lizardfish and monk seals. The story is beautifully illustrated with ink and water colours, showing microbes and chemical reactions labelled unobtrusively with scientific terms and taxonomic names in non-intimidating, handwritten script. The text tells a compelling story for a young audience but leaves many aspects of the drawings unexplained, giving opportunity for a teacher or a knowledgeable parent to embellish on the biology and answer questions about what is depicted. It anthropomorphises the characters – such as bacteria running for their lives from threatening protozoa, and the Great Guardian Haemocytes with their monocular eye (a nucleus, of course), but this is harmless fun and should hold a young listener's attention. The characters have scientifically meaningful names, such as Ali, Spiri and Sepio, which can also lead to further discussion.

The 28-page story is aimed at a primary school audience, and it is followed by a 13-page section on the underlying science that could be appreciated by advanced primary school or junior secondary school students. Topics such as symbiosis, squid biology, quorum sensing, genetics, classification and the chemistry of bioluminescence as well as a glossary are explained lightly and concisely, with ample illustrations in the same colourful, story book vein. Similar to the authors' other delightful book (*Zobi and the Zoox; A Story of Coral Bleaching*), the story emphasises mutualistic symbiosis, not just its importance in evolution and ecology but also as a lesson for children about cooperation. The book is a beautiful, engaging, and effective work of science communication for a young audience.

Also valuable are the publisher's online notes for teachers. The notes include learning objectives, quizzes for kids, thought-provoking questions for discussion, drawing activities, related learning activities, and links to relevant curriculum resources.

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Future issues of *Microbiology Australia*

May 2019: Antimicrobial resistance
Guest Editors: Jonathan Iredell, John Merlino and Andrew Ginn

September 2019: Sustainable use and preservation of biological resources
Guest Editors: İpek Kurtböke and Wieland Meyer

November 2019: Early Career Research

March 2020: Zoonoses
Guest Editors: John Mackenzie and David Williams

May 2020: Probiotics
Guest Editor: Hanna Sidjabat

September 2020: Advanced microbiology imaging and microbiology methods

November 2020: Tier 1 and 2 security sensitive organisms

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Australian Journal of Zoology
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Australian Mammalogy
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Australian Systematic Botany
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Environmental Chemistry
Chemical approaches to environmental problems

Functional Plant Biology
Plant function and evolutionary biology

Historical Records of Australian Science
The history of science, pure and applied, in Australia and the southwest Pacific

International Journal of Wildland Fire
The journal of the International Association of Wildland Fire

Invertebrate Systematics
Systematics, phylogeny and biogeography

Journal of Primary Health Care
The journal of The Royal New Zealand College of General Practitioners

Marine and Freshwater Research
Advances in the aquatic sciences

Microbiology Australia
The journal of the Australian Society for Microbiology

Pacific Conservation Biology
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Rubbo Oration Speaker:

Prof Tilman Ruff

University of Melbourne, AUS

Invited Plenary Speakers:

Dr Marnie L Peterson

University of Wyoming, USA

A/Prof Noah Fierer

University of Colorado, USA

Great Scientific & Social Program already in place