3rd International hands-on PHAGE BIOTECHNOLOGY course

Handbook

JUNE 19-23, 2017 | BRAGA-PORTUGAL

WELCOME MESSAGE

Dear participants,

It is my great pleasure to welcome you to **the 3rd International hands-on PHAGE BIOTECHNOLOGY course**. This course is organized by the Bacteriophage Biotechnology Group at the University of Minho every two years since 2013. During the last 6 years phage biotechnology has witnessed many scientific advances, and new emerging tools are having a great contribution to the field.

The three full days of hands-on experiments will focus on basic and cutting edge topics of phage biotechnology such as: phage isolation and characterization, genome annotation, expression and characterization of phage-related proteins, and phage display.

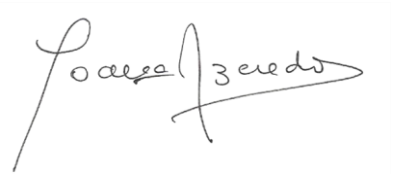
We are honored to have a speaker panel of excellent internationally recognized phage researchers from Academia and Industry that will deal with a broad range of subjects from phage ecology, structural analysis, phage-host interactions, omics and meta-omics phage characterization, to the application of phage and phage-derived molecules in health, veterinary science and agro-food.

To make your stay as pleasant as possible and promote interaction between participants, speakers and organizers we have prepared three social events: an outdoor "phage-hunting" team work activity, a walking tour through Braga, and a dinner at the city center.

I wish to thank the lecturers who are contributing greatly to the high scientific standard of the course. I would also like to express my gratitude to all those who, through their dedicated efforts, have assisted us in the organization of this course.

Finally I would like to wish you a fruitful and pleasant stay in Braga and hope that you will enjoy both the scientific and social events of this course.

June 2017



Joana Azeredo

ORGANIZING COMMITTEE

Joana Azeredo
jazeredo@deb.uminho.pt

Sanna Sillankorva
s.sillankorva@deb.uminho.pt

Ana Oliveira
anaoliveira@deb.uminho.pt

Ana Rita Costa
anaritamc@deb.uminho.pt

Carla Carvalho
carlacarvalho@deb.uminho.pt

Diana Priscila Pires
priscilapires@deb.uminho.pt

Hugo Oliveira
hugooliveira@deb.uminho.pt

Ivone Martins
ivone.martins@ceb.uminho.pt

Luís Melo
lmelo@deb.uminho.pt

Sílvio Santos
silviosantos@deb.uminho.pt

STAFF

Catarina Milho
catarinamilho@ceb.uminho.pt

Daniela Silva
mdanielasilva@ceb.uminho.pt

Débora Ferreira
deboraferreira@ceb.uminho.pt

Ergün Aktürk
ergun_akturk@hotmail.com

Graça Pinto
gracapinto@ceb.uminho.pt

Henrique Ribeiro
henrique.ribeiro@ceb.uminho.pt

Rodrigo Monteiro
rodrigo.m@ceb.uminho.pt

SPONSORS

The Organizing Committee gratefully acknowledges the support of the following organizations and companies:

www.alfagene.pt	www.eng.uminho.pt	www.nzytech.com
www.bioportugal.pt	www.fishersci.com	www.stabvida.pt
www.biotecnomica.pt	www.frilabo.pt	www.unicer.pt
www.deltacafes.pt	www.grisp.pt	www.vwr.com

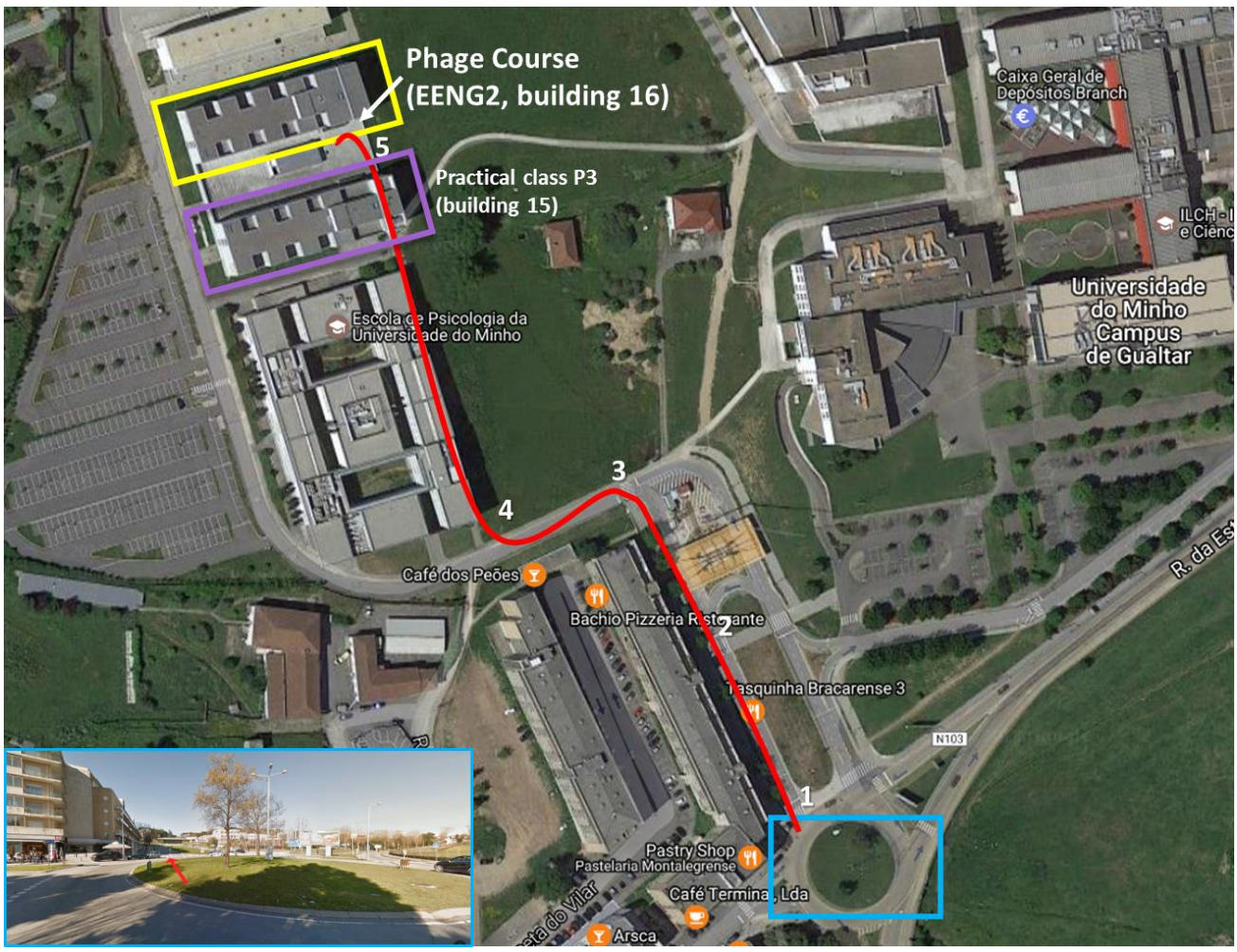
CONTENTS

GENERAL INFORMATION

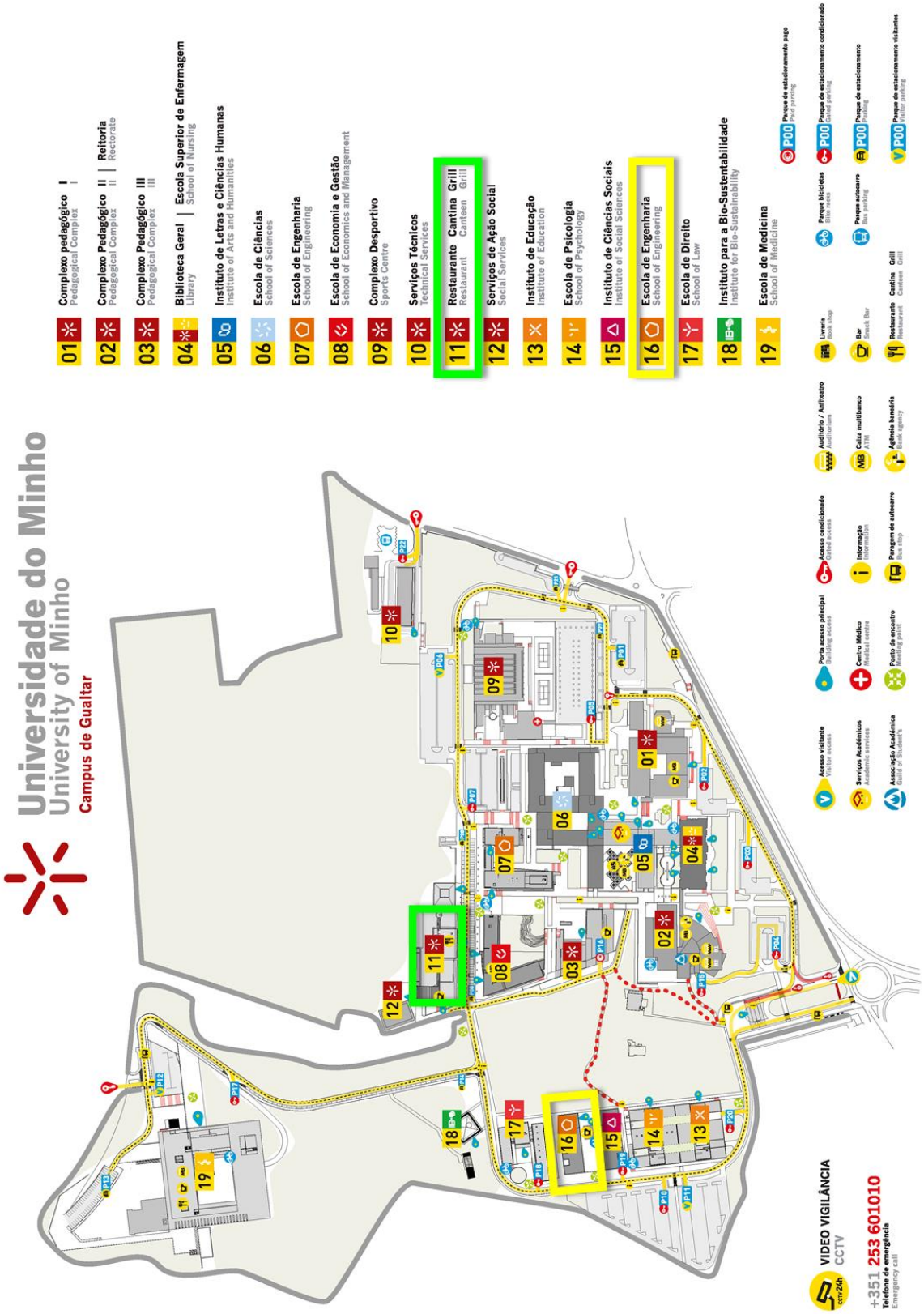
The **3rd International hands-on PHAGE BIOTECHNOLOGY course** will be held at EENG2, building 16, at Campus of Gualtar. You will find arrows on your way to get to the course building.

1. When arriving to the campus you will find an entrance close to a roundabout (**blue** square).
2. Follow the **red** line walking on the sidewalk.
3. Turn left at the end of the sidewalk.
4. Climb the stairs on your right and follow the **red** line until your reach the building (**yellow** square, building 16).
5. The entrance of the building will be indicated by the course logo.

Note: Practical Session P3 (Bacteriophage Genome Annotation) will be held at building 15 (**purple** square).



Lunch will be held every day on building 11 (**green** square). A general map of the University of Minho Campus can be seen below.



SCIENTIFIC PROGRAM

SCIENTIFIC PROGRAM AT A GLANCE

<table>
<tr><th>Time</th><th>19th June</th><th>20th June</th><th>21st June</th><th>22nd June</th><th>23rd June</th></tr>
<tr><td>9:00 - 09:45</td><td rowspan="6"></td><td>Lecture 3</td><td rowspan="2">PRACTICAL (P1)</td><td rowspan="2">PRACTICAL (P3)</td><td rowspan="2">PRACTICAL (P4/P5)</td></tr>
<tr><td>09:45 - 10:30</td><td>Lecture 4</td></tr>
<tr><td>10:30 - 11:00</td><td colspan="3">Coffee Break</td></tr>
<tr><td>11:00 - 11:45</td><td>Lecture 5</td><td rowspan="2"></td><td rowspan="2"></td><td rowspan="2"></td></tr>
<tr><td>11:45 - 12:30</td><td>Lecture 6</td></tr>
<tr><td>12:30 - 14:00</td><td colspan="3">LUNCH</td></tr>
<tr><td>14:00 - 14:45</td><td rowspan="2">Registration</td><td>Lecture 7</td><td rowspan="5">PRACTICAL (P2)</td><td rowspan="5">PRACTICAL (P3)</td><td rowspan="6">PRACTICAL (P4/P5)</td></tr>
<tr><td>14:45 - 15:00</td><td rowspan="2">Lecture 8</td></tr>
<tr><td>15:00 - 15:30</td><td>Welcome Session</td></tr>
<tr><td>15:30 - 16:15</td><td>Lecture 1</td><td>Lecture 9</td></tr>
<tr><td>16:15 - 16:45</td><td rowspan="2">Lecture 2</td><td colspan="3">Coffee break</td></tr>
<tr><td>16:45 - 17:00</td><td rowspan="3">Practical Workshop</td><td rowspan="3"></td><td rowspan="3"></td></tr>
<tr><td>17:00 - 17:30</td><td rowspan="4">Phage Hunting Activity</td></tr>
<tr><td>17:30 - 18:00</td><td rowspan="3">Farewell Party</td></tr>
<tr><td>18:00 - 18:15</td><td>Group Photo</td><td rowspan="4"></td></tr>
<tr><td>18:15 - 18:30</td><td rowspan="2">BRAGA tour</td></tr>
<tr><td>18:30 - 20:00</td></tr>
<tr><td>20:00 - 23:00</td><td></td><td>Dinner</td></tr>
</table>

DETAILED SCIENTIFIC PROGRAM

June 19, Monday		
14:00-15:00	Arrival and Registration	
15:00-15:30	Welcome Session	
15:30-16:15	Bas Dutilh	Bioinformatic approaches to discover phages and predict their hosts
16:15-17:00	Rob Lavigne	Phage genomics, proteomics and transcriptomics
17:00-17:30	Coffee Break	
From 17:30	Phage Hunting Activity	

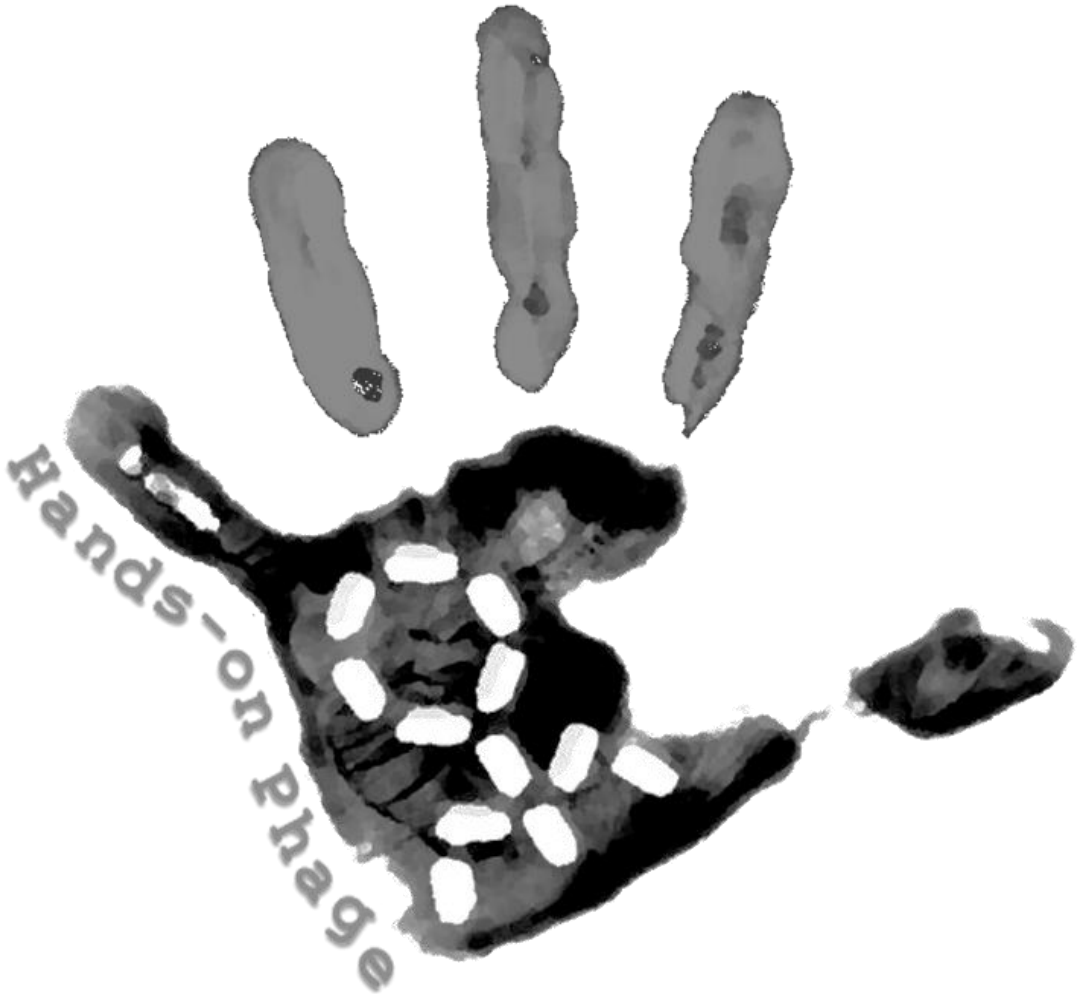
June 20, Tuesday		
09:00-09:45	Paulo Tavares	Analysis of phage particles structure and assembly
09:45-10:30	Carlos São-José	Phage-host interactions: overcoming the bacterial cell barriers
10:30-11:00	Coffee Break	
11:00-11:45	Krystyna Dabrowska	Bacteriophages in mammalian organisms
11:45-12:30	David Bikard	Exploiting CRISPR-Cas nucleases to produce antimicrobials
12:30-14:00	Lunch Break	

14:00-14:45	Manfred Biebl	Endolysins and their application
14:45-15:30	Pilar García	Phage application to agro-food
15:30-16:15	Ana Oliveira	Phage application to animal science
16:15-16:45	Coffee Break	
16:45-18:00	Practical Workshop	
18:00-18:15	Group Photo	
18:15-20:00	Braga Tour	
From 20:00	Course Dinner	

June 21, Wednesday		
09:00-10:30	Practical 1 (P1)	Bacteriophage isolation, production and titration
10:30-11:00	Coffee Break	
11:00-12:30	Practical 1 (P1) cont.	
12:30-14:00	Lunch Break	
14:00-16:15	Pratical 2 (P2)	Bacteriophage characterization
16:15-16:45	Coffee Break	
16:45-18:00	Pratical 2 (P2) cont.	

June 22, Thursday		
09:00-10:30	Pratical 3 (P3)	Bacteriophage genome annotation
10:30-11:00	Coffee Break	
11:00-12:30	Practical 3 (P3) cont.	
12:30-14:00	Lunch Break	
14:00-16:15	Practical 3 (P3) cont.	
16:15-16:45	Coffee Break	
16:45-18:00	Practical 3 (P3) cont.	

June 23, Friday		
09:00-10:30	Pratical 4 (P4) - Groups 1-4	Characterization of bacteriophage endolysins
	Pratical 5 (P5) - Groups 5-8	Phage display technology
10:30-11:00	Coffee Break	
11:00-12:30	Pratical 4 (P4) - cont.	
	Pratical 5 (P5) - cont.	
12:30-14:00	Lunch Break	
14:00-17:30	Pratical 4 (P4) - Groups 5-8	
	Pratical 5 (P5) - Groups 1-4	
17:30-18:30	Farewell Party	



LECTURE ABSTRACTS

3rd International hands-on PHAGE BIOTECHNOLOGY course

JUNE 19-23, 2017 | BRAGA-PORTUGAL

Bioinformatics approaches to discover phages and predict their hosts

Bas E. Dutilh
Utrecht University, The Netherlands
bedutilh@gmail.com

Metagenomics involves the isolation, sequencing, and analysis of genomic material from natural environments. Metagenomic datasets typically contain many unknown sequences, reflecting the undiscovered microbial diversity that has become known as “biological dark matter”. It has been hypothesized that these unknown sequences may be enriched for viruses, since viruses are traditionally more difficult to study and their genome sequences have remained under-represented in the reference databases.

To make more sense of metagenomic sequencing data, de novo sequence assembly is the method of choice to reconstruct long contigs, and several tools and approaches are available to identify phage sequences, even in total-community shotgun metagenomes dominated by cellular organisms. While in the phase of “stamp collecting” or charting the global viral sequence space, hundreds of thousands of new viral sequences have been found, and no end to the discoveries is in sight. However, follow-up questions immediately arise about the ecological role of the discovered sequences. Are they true viral genomes? Are they complete? Which host do they infect? Is it possible that they infect multiple hosts? Do they contain interesting auxiliary metabolic genes that play a role in shaping the virocell metabolism? These and similar questions we try to answer using bioinformatics. I will discuss some recent developments and challenges, and provide some handles that may be useful for your own research.

References

1. Roux S., Brum J.R., Dutilh B.E., *et al.* (2016). Ecogenomics and potential biogeochemical impacts of globally abundant ocean viruses. *Nature*, **537**: 689–693.
2. Edwards R.A., McNair K., Faust K., Raes J., Dutilh B.E. (2016). Computational approaches to predict bacteriophage-host relationships. *FEMS Microbiol Rev*, **40**: 258–272.
3. Dutilh B.E. (2014). Metagenomic ventures into outer sequence space. *Bacteriophage* **4**: e979664.
4. Dutilh B.E., *et al.* (2014). A highly abundant bacteriophage discovered in the unknown sequences of human faecal metagenomes. *Nature Communications*, **5**: 4498.

Phage genomics, proteomics and transcriptomics

Rob Lavigne
KULeuven, Belgium
rob.lavigne@kuleuven.be

In the last decade, phage research has rapidly moved into the genomics and post-genomics era. This is exemplified by the steady increase of sequenced phage genomes, the introduction of a genome/proteome based phage taxonomy and the impact of phage genomes in metavirome analyses in various ecological niches. This trend is driven by the availability of high throughput sequencing technologies. In the first part of this lecture, I will focus on emerging sequencing methods, of potential importance to phage sequencing (Illumina seq and Nanopore sequencing) and how full genome sequencing of phages translates into taxonomic classification.

In the second part of this lecture, the potential of these sequencing technologies is translated into the transcriptome analysis of phage, and the elicited bacterial transcrption response. The basic principle in using RNA sequencing technologies towards a greater understanding of phage genome organisation is explained as are strategies towards understanding the transcriptional response phage-induced 'stress' within the host.

A third and final part of the lecture delves into protein-based interactions within the phage-infected cell. Basic strategies towards protein identification (Mass spectrometry) and protein interaction analyses (*in vitro* and *in vivo* approaches) are linked to increasing our understanding of the large body of 'unknown' phage genes predicted from sequencing. From these, it is clear that phage are directing the host cell towards viral production by impact nearly every conceivable regulatory pathway and mechanism within the cell.

Analysis of phage particles structure and assembly

Paulo Tavares
I2BC, CNRS, France
paulo.tavares@i2bc.paris-saclay.fr

Viral particles (or virions) are complex structures in which the virus genetic information is surrounded by a proteinaceous capsid and sometimes also by lipids. This extracellular state of the viral cycle allows dissemination of the virus in the environment and infection of new host cells. The virion structure has two major functions. First, it protects the viral genome against environmental insult. Second, it ensures the specific recognition of the host and the efficient delivery of the virus genome to the cell to initiate intracellular multiplication of the virus. Such dual role requires that the virion is a highly robust nucleoprotein complex that, yet, is in a metastable state that ensures efficient disassembly or transfer of the viral genome to the host cell interior.

Viral particles assembly can be achieved by (**i**) co-assembly of the viral nucleic acid and the capsid protein(s) as observed in simple viruses like the tobacco-mosaic virus (TMV)[3], some animal viruses[7] or small bacterial viruses like MS2[14] and by (**ii**) formation of an empty proteinaceous container (procapsid) where the viral genome is subsequently packaged as found in numerous complex viruses[12, 18]. The latter strategy uses frequently a molecular motor to translocate the viral genome into the capsid structure. The resulting tight packing of the genome inside the viral capsid maximizes the amount of genetic information transported in the virion. It provides also a way to store energy to drive transfer of the viral genome to the host cell interior at the beginning of infection[12,13].

The assembly of viral particles follows a defined order of protein-protein, protein-nucleic acid and, in a number of cases, of protein-lipid interactions[18]. The study of these pathways and of the structure of their effector proteins identified viral lineages revealing, in a number of cases, that complex viruses infecting Archaea, Bacteria and Eukaryotes have a common ancestor[1]. This finding shows that a limited number of strategies were developed early in evolution to build successfully infectious viral particles. Assembly strategies were then elaborated during virus-host co-evolution by addition of specialized structures to ensure infection of very different hosts.

The well-characterized virion assembly pathways of the bacterial viruses (phages or bacteriophages) with a tail-herpesviruses lineage will be described to illustrate principles of virion macromolecular assembly with particular focus on phage SPP1. A strict order of macromolecular interactions leads to assembly of structures with more than 20 Mda that protect a linear double-stranded DNA (dsDNA) molecule. A procapsid is assembled first. Its capsid subunits establish quasi-equivalent interactions to build an icosahedral lattice following the Caspar and Klug rules[8], a strategy also used by structurally homologous proteins to construct cellular nanocompartments[6]. Correct positioning of the procapsid lattice subunits requires the internal scaffolding protein, a chaperone that can be an independent protein (phages P22, SPP1, phi29, herpesviruses[9]) or fused to the carboxyl terminus of the major capsid protein (phage HK97[4]). One of the 12 vertices of the icosahedron

is occupied by a specialized structure, the portal protein. Its 12 subunits are organized around a central channel through which the viral genome enters the capsid during DNA encapsidation and exits at the beginning of infection[15]. Docking of the terminase-DNA complex at the portal leads to assembly of the DNA packaging motor. The terminase is most frequently composed of two types of subunits. TerS binds the viral DNA leading to its selective encapsidation while TerL has normally endonuclease and ATPase activities[10]. Terminase-specific recognition of viral DNA is critical for assembly of infectious virus.Terminase-specific recognition of viral DNA is critical for assembly of infectious virus. The rare cases in which host DNA is packaged, transducing particles are assembled leading to horizontal gene transfer, a major mechanism of genetic exchange in bacterial populations[16]. The terminase-portal motor pumps dsDNA to the capsid interior fueled by the TerL ATPase activity. The DNA concentration can approach 500 mg/mL that applies an internal force on the capsid lattice of ~20 pN[10]. DNA packaging is normally terminated by an endonucleolytic cleavage of the substrate viral DNA defining the size of the packaged DNA. This cleavage can be sequence specific (ex. *cos* phages like lambda) or non-specific (phages packaging by a headful mechanism like T4, P22 or SPP1). The portal channel is then rapidly closed to avoid leakage of packaged DNA[15].

The late steps of virion assembly are very different in herpesviruses and in tailed phages to allow infection of animal and bacterial cells, respectively. In herpesviruses, the nucleocapsid is surrounded by a tegument and by a membrane envelope with glycoproteins that promote binding to the eukaryotic cell and subsequent membrane fusion for delivery of the nucleocapsid to the cytoplasm[5]. In tailed phages, the portal vertex of the nucleocapsid serves as a platform for assembly of a short tail (*Podoviridae*) or for attachment of long phage tails (*Siphoviridae* and *Myoviridae*). Tails are specialized structures for recognition of the host bacterium surface receptor(s) and for subsequent delivery of the naked phage genome across the bacterial envelope to reach the cytoplasm (see talk by C. São-José). Assembly of long phage tails follows a conserved pathway initiated by formation of a host adsorption/puncturing apparatus of variable complexity. This structure provides a platform to initiate helical polymerization of a tail tube whose distal end attaches to the portal vertex. Interestingly, the modules building these tails are structurally homologous to bacterial delivery machineries used in inter-bacteria warfare (bacteriocins, type VI secretion systems (T6SS)) or in bacterial attack of eukaryotic cells (T6SS)[2,17].

We will discuss the general strategies used to assemble these megadalton viral particles of homogeneous size and shape as well as their experimental study combining genetics, biochemistry, bulk and single-molecule biophysics, structural biology hybrid methods (cryo-electron microscopy, X-ray crystallography, NMR), live imaging, and comparative biology omics approaches.

References

1. Abrescia et al (2012). Structure unifies the viral universe. *Annu Rev Biochem*, **81**:795.
2. Bönemann et al (2010). Tubules and donuts: a type VI secretion story. *Mol Microbiol*, **76**:815.
3. Butler (1984). The Current Picture of the Structure and Assembly of Tobacco Mosaic Virus. *J Gen Virol*, **65**:253.
4. Hendrix, Johnson (2012). Bacteriophage HK97 capsid assembly and maturation. *Adv Exp Med Biol*, **726**:351.

5. Krummenacher et al (2013). Entry of herpesviruses into cells: the enigma variations. *Adv Exp Med Biol.*, **790**:178.

6. McHugh et al (2014). A virus capsid-like nanocompartment that stores iron and protects bacteria from oxidative stress. *EMBO J*, **33**:1896.

7. Perlmutter, Hagan (2015). Mechanisms of Virus Assembly. *Annu Rev Phys Chem*, **66**:217.

8. Prasad, Schmid (2012). Principles of virus structural organization. *Adv Exp Med Biol*. **726**:17.

9. Prevelige, Fane (2012). Building the machines: scaffolding protein functions during bacteriophage morphogenesis. *Adv Exp Med Biol*. **726**:325.

10. Rao, Feiss (2015). Mechanisms of DNA Packaging by Large Double-Stranded DNA Viruses. *Annu Rev Virol*, **2**:351.

11. Rossmann, Rao (2012). Viral Molecular Machines. *Adv Exp Med Biol*, vol. 726.

12. Santos-Pérez et al (2017). Membrane-assisted viral DNA ejection. *Biochim Biophys Acta*., **1861**:664.

13. São-José et al (2007). Pressure built by DNA packing inside virions: enough to drive DNA ejection in vitro, largely insufficient for delivery into the bacterial cytoplasm. *J Mol Biol*, **374**:346.

14. Stockley et al (2016). Bacteriophage MS2 genomic RNA encodes an assembly instruction manual for its capsid. *Bacteriophage*, **6**:e1157666.

15. Tavares et al (2012). Genome gating in tailed bacteriophage capsids. *Adv Exp Med Biol*., **726**:585.

16. Touchon et al (2017). Embracing the enemy: the diversification of microbial gene repertoires by phage-mediated horizontal gene transfer. *Curr Opin Microbiol*., **38**:66.

17. Veesler, Cambillau (2011). A common evolutionary origin for tailed-bacteriophage functional modules and bacterial machineries. *Microbiol Mol Biol Rev*, **75**:423.

18. Veesler, Johnson (2012). Virus Maturation. *Annu Rev Biophys*, **41**:473.

Phage host interactions: overcoming the bacterial cell barriers

Carlos São-José
University of Lisbon, Portugal
csaojose@ff.ul.pt

The virus particle of more than 96 % of known phages is composed of an icosahedral capsid enclosing a linear double-stranded DNA genome (dsDNA) and a tail structure attached to one vertex of the nucleocapsid (tailed phages[1]). Like all viruses, the phage genome needs to get inside host cells for replication and production of new virus particles. Likewise, at the end of infection the phage progeny has to be released to the extracellular medium to find new hosts. The bacterial cell envelope (BCE) is a protective shell made of a cytoplasmic membrane (CM), a cell wall (CW) and additionally an outer membrane (OM) in case of Gram-negative bacteria and mycobacteria[4 and references therein]. This shell is a physical barrier impenetrable by tailed phage virions. Therefore, phages evolved strategies/functions to overcome the different layers of the BCE, first to get in, and then to get out of bacteria. There is however a major difference in these two crucial steps of the phage infection cycle: virus entry cannot compromise cell viability, whereas phage particle release requires host cell lysis. This talk will present an overview of the mechanisms and protein players underlying phage DNA entry to bacteria and then virion progeny escape from infected hosts.

The phage tail plays key roles in the recognition and binding (adsorption) to host cells and then in the channeling of the viral genome into the bacterial cytoplasm[7]. Adsorption involves the specific interaction between tail receptor-binding proteins (RBPs) and one or more bacterial surface receptors. RBPs can localize in different substructures of the tail, like fibers, spikes or baseplates[3,5,9], whereas surface receptors are usually components of the outermost layers of the BCE, such as proteins, sugars and different polymeric substances[2, 12]. Appropriate tail/receptor interactions trigger the necessary virion structural rearrangements for translocation of the phage genome into host cells, which include opening of the head-to-tail connector for DNA exit and formation and insertion of a tail tube across the BCE[3,5,9]. The latter event may be assisted by virion enzymes that surgically cleave components of the BCE[8]. It has been proposed that the forces stored in the phage capsid as result of the tightly packed DNA, or the hydrodynamic forces resulting from the osmotic gradient across the CM, may push at least one initial fraction of the phage genome into cells. It has been shown for some phages that host cell energy and/or molecular motors of phage or bacterial origin are necessary for phage DNA translocation into cells[10].

Tailed phages encode two fundamental functions to lyse infected bacteria for virion progeny release: the endolysin and the holin[4,14]. Endolysins cleave the peptidoglycan, the main component of the CW, and are essential for rapid bacteriolysis. Holins are hydrophobic proteins that at the appropriate time form holes in the host cell CM, collapsing the membrane proton motive force (pmf) and leading to abrupt cell death[13]. In the so-called canonical lysis model, these holes are also essential to allow passage of the cytoplasm-accumulated canonical endolysin (c-endolysin) to the CW, thus defining the lysis timing[4,14]. In the last years, different non-canonical lysis systems have been described in which endolysins are exported to the CW compartment in a holin-independent way. In

most studied cases, endolysins engage the host general secretion pathway (the Sec system) for their export, which starts very early during the viral reproductive cycle[4,14]. Therefore, the activity of the exported endolysins (e-endolysins) needs to be contained to avoid premature bacteriolysis. It was found that mechanisms depending on an energized CM were responsible for inhibiting e-endolysins. In fact, in these systems holins still maintain the key role of defining the proper time for lysis thanks to their scheduled pmf-dissipating action, which directly or indirectly relieves the mechanisms restraining e-endolysins[4,14 and references therein]. Interestingly, boosting of endolysin activity by the holin-mediated pmf collapse seems also to occur in canonical lysis, as well as activation of host bacteria autolysins, all of which contributing to bring about fast and efficient bacteriolysis[6]. Besides the holin-endolysin dyad, tailed phages may encode accessory lysis proteins that may be important under certain physiologic conditions. Known examples are spanins and lipolytic enzymes that target the OM of Gram-negative bacteria and mycobacteria, respectively[4,11].

References

1. Ackermann H.W., Prangishvili D. (2012). Prokaryote viruses studied by electron microscopy. *Arch. Virol*. **157**: 1843-9.
2. Bertozzi Silva J., Storms Z., Sauvageau D. (2016). Host receptors for bacteriophage adsorption. *FEMS Microbiol Lett*. **363**. pii: fnw002.
3. Casjens S.R., Molineux I.J. (2012). Short noncontractile tail machines: adsorption and DNA delivery by podoviruses. *Adv Exp Med Biol*. **726**:143-79.
4. Catalão M.J., Gil F., Moniz-Pereira J., São-José C., Pimentel M. (2013). Diversity in bacterial lysis systems: bacteriophages show the way. *FEMS Microbiol Rev*. **37**:554-571.
5. Davidson A.R., Cardarelli L., Pell L.G., Radford D.R., Maxwell K.L. (2012). Long noncontractile tail machines of bacteriophages. *Adv Exp Med Biol*. **726**:115-42.
6. Fernandes S., São-José C. (2016). More than a hole: the holin lethal function may be required to fully sensitize bacteria to the lytic action of canonical endolysins. *Mol Microbiol*. 2016:92-106.
7. Fokine A., Rossmann M.G. (2014). Molecular architecture of tailed double-stranded DNA phages. *Bacteriophage*. **4**:e28281.
8. Latka A., Maciejewska B., Majkowska-Skrobek G., Briers Y., Drulis-Kawa Z. (2017). Bacteriophage-encoded virion-associated enzymes to overcome the carbohydrate barriers during the infection process. *Appl Microbiol Biotechnol*. **101**:3103-3119.
9. Leiman P.G., Shneider M.M. (2012). Contractile tail machines of bacteriophages. *Adv Exp Med Biol.* **726**:93-114.
10. Molineux I.J., Panja D. (2013). Popping the cork: mechanisms of phage genome ejection. *Nat Rev Microbiol*. **11**:194-204.
11. Rajaure M., Berry J., Kongari R., Cahill J., Young R. (2015). Membrane fusion during phage lysis. *Proc Natl Acad Sci U S A*. **112**:5497-502.
12. Vinga I., São-José C., Tavares P., Santos M.A. (2006). Bacteriophage entry in the host cell. In: Wegrzyn G. (Ed), Modern Bacteriophage Biology and Biotechnology. Research Signpost, Kerala, India, pp. 165-203.
13. Young R. (2013). Phage lysis: do we have the hole story yet? *Curr Opin Microbiol*. **16**:790-7.
14. Young R. (2014). Phage lysis: three steps, three choices, one outcome. *J Microbiol*. **52**:243-58.

Bacteriophages in mammalian organisms

Krystyna Dąbrowska
Polish Academy of Sciences, Poland
dabrok@iitd.pan.wroc.pl

Bacteriophages are parasites of bacteria, which are often symbionts or pathogens of mammals. Consequently, mammals have become an environment for phage life cycles. Phages are known to be a natural component of microbiome. They can be found in the gastrointestinal tract, saliva, or on the skin surface. Since bacteriophages can be found in food and water, mammals are constantly exposed to these viruses.

Bacteriophages may deliver important medical solutions. The first is antibacterial therapy, which makes use of the natural ability of bacteriophages to kill bacteria. Phages as an alternative to antibiotics draw scientific attention due to the problem of antibiotic resistance in bacteria. Other phage solution is the technological approach to phages as nanocarriers that are able to deliver biologically active elements. Nanocarriers may deliver many kinds of drugs, but they can also be a platform that allows for exposure of selected antigens. Such bacteriophage-based platforms are proposed as a new generation of safe (non-pathogenic) and effective vaccines.

Medical applications of bacteriophages as well as the natural exposition of human and animals to bacteriophages share a common feature: phages make a direct contact with the mammalian organism. In general phages are usually able to enter a mammalian system, but their efficiency depends on route of administration, phage type, and dose. They may transit through gastrointestinal tract, not necesserily interacting with setteled symbiotic bacteria. Phages are able to translocate from the intestine to circulation, however this is the least effective route of phage delivery, comparing to parenteral ones. Once phage gets to the blood stream, it is capable to get to virtually all mammalian organs and tissues. This gives rise to various interactions of phages with organisms of humans or animals, and these interactions determine safety issues, phage pharmacokinetics, bioavailability and resulting outcomes of this contact, including antibacterial treatment. Bacteriophages that penetrate a mammalian organism act as foreign antigens and they challenge mammalian immunological system. This may induce a humoral response to phage, but also a non-specific, innate immunity response. The type and course of immunological response to a phage depends on many factor, like phage type, dose, application route and schedule, accompanying compounds, and even immunological status of a particular individual. Immunological response is often considered undesired, like in the case of antibacterial therapy, but it can be either advantageous, like during vaccination.

Good recognition of phage interactions with mammalian organisms opens the way for both successful phage use as nanocarriers or antibacterials, and for understanding of complex balance in coexisting microbial communities.

Exploiting CRISPR-Cas nucleases to produce antimicrobials

David Bikard*, Chad W Euler, Wenyan Jiang, Philip M Nussenzweig, Gregory W Goldberg, Xavier Duportet, Vincent A Fischetti & Luciano A Marraffini
Institute,Pasteur, France
*david.bikard@pasteur.fr

Advances in DNA sequencing technologies are revealing the diversity of complex microbial populations in different environments. They are also providing evidence for the contributions that individual species make to both populations and environments. Perhaps the most striking example of this is the human microbiome and its influence on human health[1,2]. Studying the microbiome has not only shown the importance of certain species for human health[3], but has also revealed the undesired side effects of traditional antimicrobials (including antibiotics) that lack killing specificity. In addition to important negative effects on human health, antibiotic use promotes the emergence of antibiotic resistance. There is a pressing need to develop species-specific, selective antibiotics that can be used to manipulate complex microbial consortia such as the microbiome.

Cas9 (CRISPR-associated protein 9) is a double-stranded (ds)DNA nuclease present in the type II CRISPR (clustered, regularly interspaced, short palindromic repeats) immune system of bacteria that uses a 20-nt small RNA guide (the CRISPR RNA, crRNA) to specify the site of cleavage[4,5]. We and others recently showed that reprogramming the Cas9 nuclease against bacterial genomic sequences is lethal, most likely due to the introduction of irreparable chromosomal lesions[6,7]. This observation led us to explore the possibility of using this nuclease as a sequence-specific antimicrobial, a tool that would allow selective killing of one or more bacterial species within a heterogeneous population. To achieve this, the type II CRISPR system has to be delivered to as many target cells as possible (if not all), without the need for selection, and in a manner that can easily be used to treat bacterial populations in their natural environment. Bacteriophages naturally package their DNA into capsids, which can then inject their content into host bacteria. Therefore we opted to deliver the *cas9* gene and its RNA guide/s sequences using a phagemid, which is a plasmid that is designed to be packaged in phage capsids[8]. This strategy was in part inspired by the recent discovery of a phage carrying its own CRISPR system[9].

We show that Cas9, reprogrammed to target virulence genes, kills virulent, but not avirulent, Staphylococcus aureus. Reprogramming the nuclease to target antibiotic resistance genes destroys staphylococcal plasmids that harbor antibiotic resistance genes and immunizes avirulent staphylococci to prevent the spread of plasmid-borne resistance genes. We also show that CRISPR-Cas9 antimicrobials function in vivo to kill S. aureus in a mouse skin colonization model. This technology creates opportunities to manipulate complex bacterial populations in a sequence-specific manner.

References

1. Round J.L. et al. (2011) The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science* **332**: 974-977.
2. Mazmanian S.K., Round J.L., Kasper D.L. (2008) A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* **453**: 620-625.

3. Dethlefsen L., Huse S., Sogin M.L., Relman D.A. (2008) The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS Biol* **6**: e280.
4. Jinek M. et al. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**: 816-821.
5. Gasiunas G., Barrangou R., Horvath P., Siksnys V. (2012) Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc. Natl. Acad. Sci. U.S.A.*
6. Gomaa A.A. et al. (2013) Programmable removal of bacterial strains by use of genome-targeting CRISPR-Cas systems. *mBio* **5**: e00928-00913.
7. Jiang W., Bikard D., Cox D., Zhang F., Marraffini L.A. (2013) RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nat Biotechnol* **31**: 233-239.
8. Melnikov A.A., Tchernov A.P., Fodor I., Bayev A.A. (1984) Lambda phagemids and their transducing properties. *Gene* **28**: 29-35.
9. Seed K.D., Lazinski D.W., Calderwood S.B., Camilli A. (2013) A bacteriophage encodes its own CRISPR/Cas adaptive response to evade host innate immunity. *Nature* **494**: 489-491.

Endolysins and their application

Manfred Biebl
Lisando GmbH, Germany
manfred.biebl@lisando.com

At the end of the lytic life cycle the progeny of bacteriophages has to be released from their host bacterium. A complex system mostly consisting of peptidoglycan degrading enzymes (endolysins) supported by holins, proteins allowing endolysins to pass the inner membrane, is responsible for this final step in phage amplification. Endolysins itself show a wide range of variation within structure and target. Whereas endolysins of phages specific for Gram-negative bacteria mostly show globular structure, the lysins specific for Gram-positive bacteria (e.g. *Staphylococcus* sp.) exhibit a modular composition. Besides an enzymatically active domain (EAD), responsible for the degradation of the peptidoglycan, these endolysins possess so-called cell wall binding domains (CBDs). Based on their cleavage site within the peptidoglycan, EADs can be divided in several classes (glycosidases, amidases and endopeptidases). The modular structure implicates advantages which can be used in several applications. The specificity of CBDs is very useful for the development of diagnostic tools, because they can be fused to reporter proteins (e.g. GFP) or linked to surfaces (e.g. magnetic beads). Another advantage is the possibility of combining and exchanging the single modules to create new lyins with varying characteristics. By this way lytic enzymes can be designed and adopted for specific requirements concerning targeted bacteria and environments. During the last years, many scientific groups and companies used this technology to develop lysins for different applications, like skin treatment or even bacteremia. Unfortunately, this technologies were always limited to Gram-positive bacteria, as Gram-negatives are protected by an outer membrane, which prevents the peptidoglycan being attacked from the outside. Recent progress in technology allows nowadays to modify endolysins specifically for Gram-negative bacteria to overcome this hurdle. Therefore, it is possible to create urgently needed special designed endolysins (Artilysin®) fighting Gram-negative pathogens like *Pseudomonas aeruginosa*, *Acinetobacter baumannii* or *Enterobacteriaceae*.

References

1. Schmelcher M. et al. (2012). Bacteriophage endolysins as novel antimicrobials. *Future Microbiol.* **7**:1147-71.
2. Briers Y., Lavigne R. (2015) Breaking barriers: expansion of the use of endolysins as novel antibacterials against Gram-negative bacteria. *Future Microbiol.* **10**:377-90.

Phage application to agro-food

Pilar García
Dairy Research Institute of Asturias, Spain
pgarcia@ipla.csic.es

Foodborne diseases represent a high social and economic impact for health care systems and food industries worldwide. For instance, the economic losses in the food industry associated to infectious diseases have been estimated at over 75 billion dollars per year in the US alone. In addition, it is well known that the widespread use of antimicrobials in agri-food production has favored the increase in antimicrobial resistance in bacteria leading to the current global health crisis[8]. Bacteriophages offer some advantages over classical systems to control pathogenic bacteria throughout the food chain. Phages are highly specific for their target bacteria, innocuous for humans, environmentally friendly and have no effects on the organoleptic properties of foods[12].

Notably, bacteriophages may have a versatile use throughout the food chain from primary production to final product. Phages have been successfully used to control the most important pathogens such as *Listeria monocytogenes*, *Salmonella*, *Escherichia coli* O157:H7, *Campylobacter jejuni* and *Staphylococcus aureus*. Overall, bacteriophages have been proven to control plant pathogenic bacteria, to reduce colonization by zoonotic bacteria in farms, to decontaminate raw products, to remove biofilms from industry equipment and food-contact surfaces, and to inhibit the growth of pathogenic bacteria in foods. The approval by the USFDA of phage-based products against *L. monocytogenes*, *E. coli* O157:H7 and *Salmonella* had boosted the interest in these applications. However, the highly restrictive European regulatory framework is delaying the extensive use of phage-based products in EU agri-food industries.

The research carried out by our team is focused in *S. aureus*, one of the main bacteria causing mastitis in cows, and an important food-borne pathogen. We have isolated and characterized four bacteriophages (phiIPLA88, phiIPLA35, phiIPLA-RODI and phiIPLA-C1C) and studied their potential to control the target bacteria in dairy products (biopreservatives)[1,2,10] and in preformed biofilms (disinfectants)[4,5,7]. Moreover, phage lytic proteins (endolysins and virion-associated peptidoglycan hydrolases)[3,9,11] and phage encoded depolymerases[6] have been explored as new tools to improve Food Safety.

References

1. Bueno E., García P., Martínez B., Rodríguez A. (2012). Phage inactivation of *Staphylococcus aureus* in fresh and hard-type cheeses. *Int J Food Microbiol*, **158**:23-27.
2. García P., Madera C., Martínez B., Rodriguez A. (2007). Biocontrol of *Staphylococcus aureus* in curd manufacturing processes using bacteriophages. *Int Dairy J*, **17**:1232-1239.
3. García P., Martínez B., Rodríguez L., Rodríguez A. (2010). Synergy between the phage endolysin LysH5 and nisin to kill *Staphylococcus aureus* in pasteurized milk. *Int J Food Microbiol*, **141**: 151-155.
4. Gutiérrez D., Ruas-Madiedo P., Martínez B., Rodríguez A., García P. (2014). Effective removal of staphylococcal biofilms by the endolysin LysH5. *PlosOne*, **9**:e107307.

5. Gutiérrez D., Vandenheuvel D., Martínez B., Rodríguez A., Lavigne R., García P. (2015a). Two phages, phiIPLA-RODI and phiIPLA-C1C, lyse mono- and dual-staphylococcal biofilms. *Appl Environ Microbiol*, **81**: 3336-3348.

6. Gutiérrez D., Briers Y., Rodríguez-Rubio L., Martínez B., Rodríguez A., Lavigne R., García P. (2015b). Role of the pre-neck appendage protein (Dpo7) from phage vB_SepiS-phiIPLA7 as an anti-biofilm agent in staphylococcal species. *Front Microbiol*, **6**:1315.

7. Gutiérrez D., Rodríguez-Rubio L., Martínez B., Rodríguez A., García P. (2016). Bacteriophages as weapons against bacterial biofilms in the food industry. *Front Microbiol*, 8;7:825.

8. Koike S., Mackie R., Aminov R. (2017). Agricultural use of antibiotics and antibiotic resistance. In book: Antibiotic Resistance Genes in Natural Environments and Long-Term Effects, Chapter: Chapter 8, Publisher: Nova Science Publishers, Editors: Salvador Mirete, Marcos López Pérez.

9. Obeso J., Martínez B., Rodriguez A., García P. (2008). Lytic activity of bacteriophage ΦH5 endolysin against *Staphylococcus aureus* in milk. *Int. J. Food Microbiol*, **128**: 212-218.

10. Obeso, García P., Martínez B., Arroyo-López F. N., Garrido-Fernández A., Rodriguez A. (2010). Use of logistic regression for prediction of the fate of *Staphylococcus aureus* in pasteurized milk in the presence of two lytic phages. *Appl Environm Microbiol*, **76**: 6038-6046.

11. Rodríguez-Rubio L., Martínez B., Donovan D. M., García P., Rodríguez A. (2013). Potential of the virion-associated peptidoglycan hydrolase HydH5 and its derivative fusion proteins in milk biopreservation. *PlosOne*, **8**:e54828.

12. Sillankorva S.M., Oliveira H., Azeredo J. (2012). Bacteriophages and their role in food safety. *Int J Microbiol*, **2012**:863945.

Phage application to animal science

Ana Oliveira
University of Minho, Portugal
anaoliveira@deb.uminho.pt

Antibiotic resistance is an important threat to human health and in part it is related to the misuse of antibiotics in animal production, mainly in intensive production systems. Accordingly, animal production is facing some difficulties due to restriction in antibiotic usage and the lack of new active antimicrobial ingredients.

Biotechnology-derived solutions, in particular bacteriophages and their derived products are being considered valuable antimicrobial alternatives and their application in animal production constitutes an important strategy towards the reduction of antibiotics usage in agro-food and the increase in animal productivity, providing environmental protection.

Bacteriophages (phages) are naturally occurring predators of bacteria, ubiquitous in the environment, with high host specificity and harmless to animals. Several *in vivo* studies support the use of phages as an intervention strategy for reducing diverse bacterial infections in food-producing animals. For example, poultry industry is highly affected by the Avian pathogenic *Escherichia coli* (APEC); dairy cattle deals with the problem of mastitis mainly caused by *Staphylococcus aureus*; pigs' respiratory diseases are the most important health concern for pig producers nowadays due to bacterial pathogens like *Actinobacillus*, *Bordetela*, *Haemophilus*, etc. In the case of APEC, we have shown that crude phage lisates are not toxic to chickens and that the administration by spray and in the drinking water and can confer protection to respiratory infections. We have tested a cocktail of three phages in large production units and demonstrated that the cocktail was able to significantly reduce chicken mortality avoiding the spread of the disease.

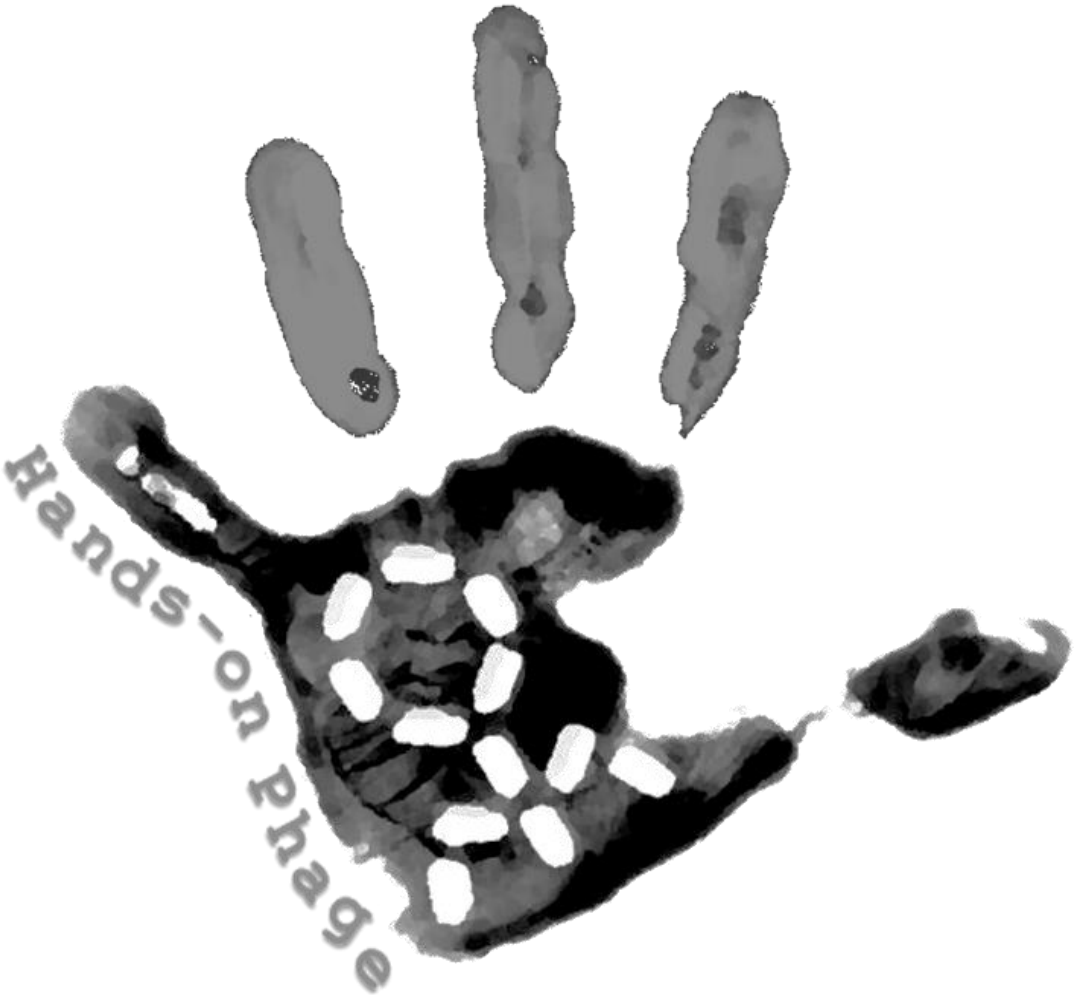
Recently, we have explored the potential use of phages for controlling American Foulbrood in honeybees, whose agent is *Paenibacillus larvae*. As it is not allowed to use antibiotics in honey production, this disease is controlled by the complete destruction of the hive through burning, resulting in great loses for the honey industry. We have isolated and characterized several *P. larvae* infecting phages. Most of the phages are temperate and therefore their use was carefully evaluated. Presently, we are curing phage producing strains and genetically manipulating the phages to make integrases dysfunctional. Simultaneously, we have performed phage bio-distribution assays to understand if phages can reach young larvae (bacteria target) when administered in the drinking water to adult bees.

Currently, regulatory bodies such as the US Food and Drug Administration (FDA), the US Department of Agriculture (USDA) and the European Medicines Agency (EMEA) have shown some flexibility in approving the use of phages for control of several problematic pathogens. In addition, there are already several companies developing and commercializing phage products that can be used in Veterinary Medicine such as BioPhage-PA, a topical phage product for chronic otitis against antibiotic-resistant *Pseudomonas aeruginosa* and Viridax for the treatment of respiratory, systemic and topical infections of *Staphylococcus aureus* and other Staphylococcal species. Therefore, phage

therapy in animal production is starting to gain importance and should be further explored.

References:

1. Carvalho C., Costa A.R., Silva F., Oliveira A. (2017). Bacteriophages and their derivatives for the treatment and control of food-producing animal infections. *Critical reviews in Microbiology*, **45**(5):583-601.
2. Oliveira A., Leite M., Kluskens L.D., Santos S.B., Melo L.D.R., Azeredo J. (2015) The First *Paenibacillus larvae* Bacteriophage Endolysin (PlyPl23) with High Potential to Control American Foulbrood. *PLoS ONE*, **10**(8): e0136331.
3. Oliveira A., Melo L.D.R., Kropinski A.M., Azeredo J. (2013). Complete genome sequence of the broad-host-range *Paenibacillus larvae* phage phiIBB_Pl23. *Genome Announcements*, **1**(5):e00438-13.
4. Oliveira A., Sereno R., Azeredo J. (2010) *In vivo* efficiency evaluation of a phage cocktail in controlling severe colibacillosis in confined conditions and experimental poultry houses. *Veterinary Microbiology*, **146**:303-308.
5. Oliveira A., Sillankorva S., Quinta R., Henriques A., Sereno R. Azeredo, J. (2009). Isolation and characterisation of bacteriophages for avian pathogenic *e. coli* strains. *Journal of Applied Microbiology*, **106**(6): 1919-1927.
6. Oliveira A., Sereno R., Nicolau, A., Azeredo, J. (2009). The influence of the mode of administration in the dissemination of three coliphages in chickens. *Poultry Science*, **88**(4): 728 - 733.
7. Oliveira A., Sereno R., Nicolau, A., Azeredo, J. (2009). *In vivo* toxicity study of phage lysate in chickens. *British Poultry Science*, **50**(5): 558—563.



POSTER ABSTRACTS

3rd International hands-on PHAGE BIOTECHNOLOGY course

JUNE 19-23, 2017 | BRAGA-PORTUGAL

Structural and functional stabilization of bacteriophage particles in biopolymeric matrices for *Pseudomonas aeruginosa* biosensing: bacterial diffusion studies

A.C. Rios[1], F.S. Del Fiol[1], M.M.D.C. Vila[1], R. Lima[1], M. Tubino[3], V.M. Balcão[1,2,*]
[1]University of Sorocaba, Brazil, [2]University of Minho, Portugal, [3]University of Campinas, Brazil
*victor.balcao@prof.uniso.br

Over recent past years, bacteriophage (or phage) research has experienced a renaissance especially with the increase of bacterial resistance to antibiotics and the possibility for phage particles to integrate new methods of early detection and diagnosis of bacterial infections. In this context, the structural and functional stabilization of phage particles within biopolymeric microporous hydrogel matrices represents a promising research focus with a broad potential biomedical/biopharmaceutical application, together with the need for a detailed knowledge of the bacterial diffusion profile into the hydrogel core, where the phage particles lie.

This research work aimed both at developing biopolymeric, non-toxic, phage-hydrogels of agar and sodium alginate, obtained at neutral pH and mild polymerization conditions, in order to structurally and functionally stabilize phage particles with maintenance of their lytic activity, and to discover which should be the characteristics of the hydrogels in order to optimize their responses as devices for biodetection of *Pseudomonas aeruginosa.* For this, a mathematical model was developed and validated experimentally, allowing prediction of both the bacterial concentration profile and the fraction of total bacteria incorporated into the hydrogels as a function of hydrogel porosity, thickness and pore constrictivity.

Disc-like phage-hydrogels were prepared, with a phage and polymer concentration of $1.3x10^8$ PFU/mL and 1.5 % (w/v), respectively. Regarding the alginate hydrogels, $CaCO_3$ (22.5 mM) and GDL (48 mM) were also included in the formulation. Agar hydrogels were prepared naturally by gellification, as a function of temperature lowering, and alginate hydrogels were prepared by internal gelation. The matrices were inoculated with a suspension of susceptible (host) bacteria and incubated at 37ºC for 24h, for detection of phage lytic activity. Subsequently, the (optimized) hydrogels were fixed to a 3D-matrix support, leaving a 2D-surface which was exposed to a (fixed volume of) bacterial suspension. Assumptions underlying development of the mathematical model encompassed (i) plane geometry, (ii) thin hydrogel film, and (iii) consideration of unidirectional diffusion. By disregarding the reaction between bacterium and phage particles, this assumption allowed to evaluate the penetration of bacteria into the biopolymeric matrix for the optimization of contact time to achieve bacterial detection. The only driving force for bacterial diffusion considered in this research effort was a cell concentration gradient. Observation of lysis on the bacterial lawn demonstrated that phage particles kept their lytic activity, being the method of physical entrapment able to promote their stabilization. Both types of phage-hydrogels presented an interconnective microporous network, ensuring a facilitated access of the phages to the bacteria, thus promoting an efficient lysis of the host bacteria present in the surface of the hydrogels.

The postulated model was able to accurately describe the evolution of both the bacterial concentration profile in the hydrogel core as well as the fraction of total bacteria embedded in both agar and calcium alginate hydrogels. From the non-linear fittings made to the experimental data, the effective diffusion coefficients for *P. aeruginosa* in agar hydrogel ($D_{bact,eff,agar} = 2.456 \times 10^{-11}$ m^2/s) and in calcium alginate hydrogel ($D_{bact,eff,Ca\text{-}alginate} = 1.290 \times 10^{-10}$ m^2/s) were obtained. Using these diffusion coefficients of bacteria in the agar and calcium alginate hydrogels in the mathematical model, the (predicted) trend in the concentration profiles along the z coordinate of the hydrogels allowed to conclude that it takes about 5 hours to cells of *P. aeruginosa* to penetrate 3 mm deep within the agar hydrogel core, while this time is reduced to only 1 hour in the case of calcium alginate hydrogel. It can thus be inferred that a biosensing device using the calcium alginate hydrogel will respond more quickly.

The porosity has a marked effect on the ability of bacteria to penetrate in the hydrogels. The pore constrictivity is not so decisive, but when it varies from 0.80 to 0.95 an increase of 6 % in the fraction of bacterial cells incorporated can be observed.

Acknowledgements

Project funding by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, São Paulo, Brazil; Refs. No. 2016/08884-3 (Project PneumoPhageColor) and 2016/12234-4 (Project TransAppIL)), is hereby gratefully acknowledged. This work also received support from CNPq, National Council for Scientific and Technological Development – Brazil, in the form of a Research Productivity (PQ) fellowship granted to Victor M. Balcão (Ref. No. 306113/2014-7).

Phage-bacteria evolutionary dynamics and how it affects phage therapy

Zeinab Hosseini-Doust
McMaster University, Canada
doust@mcmaster.ca

The rise of bacterial variants in the presence of lytic phage has been one of the basic grounds for evolution studies. However there are incongruent results among different studies investigating the effect of acquiring phage resistance on bacterial fitness and virulence. We employed an in vitro burn wound model to study the development of phage resistance and associated variations in virulence and biofilm formation for the resulting phage-resistant PA phenotypes. Experimental evolution was used to generate Pseudomonas aeruginosa PAO1 variants under selective pressure from different homogeneous and one heterogeneous phage environment. The phages were chosen to target different receptors to decrease the chance of cross-resistance.

Investigation of phenotypic traits of the variants revealed significant changes in various fitness and virulence determinants such as growth, motilities, biofilm formation, resistance to oxidative stress and production of siderophores and chromophores compared to the control. The choice of therapeutic phage (or phage cocktail) allowed for the control of evolution of resistant phenotypes (i.e., selective pressure). This work is currently being followed up by in vivo experiments on various models and also using microfabricated microenvironments for a more mechanistic investigation.

The knowledge gained from this study will fundamentally contribute to our understanding of the evolutionary dynamics of bacteria under phage selective pressure, which is crucial for the efficient utilization of bacteriophages for phage therapy.

The protein-protein interaction study between BFK20 replication protein gp41 (helicase), truncated gp41dC and the *B. flavum* CCM 251 replication proteins using BACTH

Adela Tkáčová*, Nora Halgašová, Gabriela Bukovská
Slovak Academy of Sciences, Slovak Republic
*adela.tkacova@savba.sk

Bacteriophages are viruses that infect bacteria with high rate of specificity and efficiency. Interactions between phage-encoded and bacterial proteins inside the infected cell are important for efficient infection of the host cell. Using these interactions the bacteriophage directs the host-cell metabolism towards phage directed. During the infection cycle the phage could have an impact on the bacterial transcription, translation or replication machinery. An important part of the study of phage replication mechanisms is determining what replication proteins are present in the phage replisome and what their interactions are. Most of the proteins involved in the phage replisome are of phage origin (e.g. T4, T7)/or are host replication proteins modified by specific phage proteins. The widely used method of study protein-protein interactions *in vivo* is two-hybrid screening. Using bacterial two hybrid system - BACTH for testing protein-protein interactions we previously determined significant interactions between the BFK20 protein gp41 (a putative SF2 helicase) and the B. flavum CCM 251 replication proteins DnaZX (gamma/tau subunit of DNA polymerase III), DnaN (beta subunit of DNA polymerase III), Dnaδ (delta subunit of DNA polymerase III), SSB (single strand binding protein) and DnaG (primase). According our results we also concluded that the replication module of BFK20 is likely to be of type 2 and gp41 together with DnaG and probably along with other tested host replication proteins participate in BFK20 DNA replication.

In our present study we tested protein-protein interactions between the BFK20 protein gp41 and other three replication proteins of *Brevibacterium flavum* CCM 251: DnaE1 (alpha subunit of DNA polymerase III), DnaJ1 (chaperon protein), DnaLig (DNA ligase) using BACTH system as well. To elucidate the region on protein gp41 responsible for the interactions between molecules of proteins we prepared truncated protein gp41dC with removed C-terminal region. In next we tested protein-protein interactions between gp41, truncated protein gp41dC and the eight B. flavum CCM 251 replication proteins using bacterial two hybrid system BACTH. Beta-galactosidase activity measurements showed that the interaction between gp41 and host proteins are stronger compare to interactions between gp41dC and tested host proteins. We conclude that C-terminal region of protein gp41 is most probably involved in protein-protein interaction mechanism.

Acknowledgements
This work was supported by the VEGA Grant 2/0122/14 from Slovak Academy of Sciences.

Evaluation of Sb-1 and Pyo-bacteriophage activity to treat and prevent Staphylococcus aureus biofilm formation by isothermal microcalorimetry

Mariagrazia Di Luca*, Tamta Tkhilaishvili, Andrej Trampuz
Charité - University Berlin, Germany
*mariagrazia.di-luca@charite.de

Biofilms on both soft tissue and implants are causing persistent infections that are often difficult to eradicate, also due to the fact that microbial cells in biofilm are more resistant to antibiotics than their planktonic counterparts. As a result, new strategies to fight biofilm infections need to be considered. Bacteriophages have emerged as a promising alternative because of their high selectivity and rapid bactericidal activity, which they also exert on multi-resistant bacteria. In this study we investigated the activity of lytic bacteriophages against methicillin resistant *Staphylococcus aureus* (MRSA) in planktonic and biofilm form by taking advantage of the high sensitivity of isothermal microcalorimetry.

S. aureus specific phage (Sb-1) and Pyo-bacteriophage cocktail, were tested against methicillin-resistant MRSA ATCC 43300 and different clinical strains isolated by prosthetic joint infections. For eradication studies, MRSA was co-incubated for 24h at 37 °C in BHI broth (BHIB) with porous glass beads in order to allow biofilm formation. The beads were then washed and, exposed to different titers of bacteriophages. After a second wash, treated beads were placed in sealed ampoules containing BHIB. The heat flow (µW) and total heat (J) were measured in real-time for 48h by isothermal microcalorimetry. In biofilm prevention experiments, MRSA was directly co-incubated with different bacteriophage titers and glass beads for 24h at 37 °C, and contextually monitored by microcalorimetry analysis.

Both Sb-1 and Pyo-phage bacteriophages exerted a dose-dependent anti-biofilm activity on MRSA, as attested by the inhibition of the heat production observed within the first 24h. However, MRSA biofilm was eradicated only by co-incubation with the highest phage titer tested (10^7 PFUs/ml for Sb-1 and 10^6 PFUs/ml for Pyo-phage). Notably, after a 48h-exposure, also the lowest titers tested resulted in a strong reduction of biofilm viability. By contrast, a significant reduction of MRSA heat production was already achieved at a lower titer (10^2 PFUs/ml) for both bacteriophages in prevention experiments, and the presence of 10^4 PFUs/ml completely abolished the heat production. Hence, bacteriophages might interfere with the early stages of MRSA biofilm formation (e.g. adhesion on the glass beads).

Our results showed that Sb-1 and Pyo-phage are able to both eradicate MRSA biofilm *in vitro* and prevent its formation, suggesting that phage therapy may be a promising approach for preventing device colonization and controlling biofilms on surface. Novel strategies for direct coating of biomaterials with bacteriophages and/or local release of phages at the site of the infection might also be further investigated.

Bacteriophages for a green wine

Ifigeneia Kyrkou*, Alexander BythCarstens and Lars Hestbjerg Hansen
Aarhus University, Denmark
*ifigeneia.kyrkou@envs.au.dk

Consumers' demand for wine of enhanced organoleptic attractiveness that should result from sustainable practices during all stages of wine making is increasing the challenges for the wine industry. Thus, the development of efficient biological treatments is more than urgent and relevant, so as to control the bacteria that are affecting the final quality of wine.

In light of these, the aim of our ongoing study is to elucidate the role of bacteriophages in promoting or impeding bacteria important for the wine production and to investigate the application of phage treatment to shape the sensory character of the final product.

For this reason, special focus is given on finding bacteriophages against the traditional malolactic fermentation starter, *Oenococcus oeni*. Furthermore, the existence of potential bacteriophage foes of *Lactobacillus plantarum* -a bacterium participating in the malolactic fermentation of wine and having a great potential as a new generation starter- is investigated. The aforementioned tests are being conducted using a wide range of samples.

Basic characterization of two *S. aureus* bacteriophages isolated from waste water

D. Stverakova[2-3]*, L. Fisarova[2-3], K. Rosikova [3], M. Benesik[2-3], M. Siborova[1], P. Plevka[1], R. Pantucek[2], M. Mosa[3]

[1] Central European Institute of Technology, [2] Masaryk University, [3] MB Pharma; Czech Republic

*dana.stverakova@gmail.com

Staphylococcus aureus is commensal and opportunistic pathogen and it is also a major causative agent of hospital-associated infections. Antibiotic resistant strains have emerged as a significant threat in hospital and community environment and a method of choice in curing such infections might be phage therapy.

In this work we describe two newly isolated phages infecting *S. aureus*. Phages were isolated from samples of waste water originating from Brno and České Budějovice. Both phages were propagated on prophage-less *S. aureus* strain and showed clear plaques. Using PCR (Pantůček, *et al*. 2004) the phages were identified as members of the *Myoviridae* family, Twort-like, belonging to serological group D. The morphology of bacteriophages was determined by electron microscopy. Size of the phage genomes was estimated according to restriction profiles. Virion proteins of the newly isolated phages were compared using SDS-PAGE.

We analyzed the lytic host range and lytic ability of both phages using spot test on a collection of laboratory strains and clinical isolates including MRSA strains. Lytic ability of the newly isolated phages was compared to commercial preparation Stafal and polyvalent staphylococcal phage 812, which is known for its wide host range. Both new phages proved to be promising candidates for the therapeutic use. Our aim is to create a phage cocktail for curing staphylococcal infections in humans and animals.

Acknowledgements

We kindly acknowledge the financial support: CZ.01.1.02/0.0/0.0/15_019/0004353.

Staphylococcal phages A3R and 676Z induce IgG production in oral and intraperitoneal administration in a murine model with a contribution of major capsid protein

Joanna Majewska*, Zuzanna Kaźmierczak, Karolina Wojtyna, Dorota Lecion, Katarzyna Hodyra-Stefaniak, Anna Kłopot, Paulina Miernikiewicz, Marek Harhala, Barbara Owczarek, Katarzyna Zielonka, Andrzej Górski, Krystyna Dąbrowska
Polish Academy of Sciences, Poland
*joanna.majewska@iitd.pan.wroc.pl

Bearing in mind a major role of the immune responses for phage activity *in vivo*, we investigated two therapeutic staphylococcal bacteriophages: A3R and 676Z regarding their ability to induce IgG production and the contribution of their major capsid protein to this effect. C57Bl6/J mice were treated with purified phage preparations orally or intraperitoneally. For oral application, animals were given phage preparations in PBS, mixed with drinking water (1:1) to a final concentration of 4×10^9 pfu/ml continuously for 100 days. Phages were then removed from the diet and the experiment was continued for the next 50 days. Control group was given drinking water with PBS. For intraperitoneal immunization, mice were injected with phage preparations 10^9 pfu/mice on day 1, 20 and 50, while control mice were injected with PBS. In both cases blood samples were collected throughout the experiment and evaluated for the presence of anti-phage IgG antibodies by ELISA immunoassay. Plasma samples collected on day 100 in both immunizations were then tested for IgG antibodies specific for major capsid protein (AFN38122.1 / AFN38316.1), which shares 100 % amino acid sequence homology in both phages. Structural function of the protein was confirmed using TEM: antibodies from mice immunized s.c. with AFN38122.1 protein were precipitated, conjugated with gold nanoparticles, incubated with phage samples and then visualized.

In both models significant induction of anti-phage IgG antibodies was observed. Oral administration of phage preparations resulted in a marked increase of plasma IgG within two weeks following the initiation of the treatment. In case of i.p. route of immunization, IgG levels started to increase sooner, on day 3 after first injection. Once induced, IgG levels continued to rise until day 22-29 and remained at high concentration long after the last doses of phage preparations were injected or after oral phage treatment was terminated. IgG antibodies specific to the major capsid protein AFN38122.1 were detected in plasma samples collected on day 100 for both routes of administration. Fold of increase in ELISA units after oral application reached approximately 142 for A3R and 106 for 676Z, while in case of i.p. immunization it reached 319 for A3R and 199 for 676Z. These data show that staphylococcal bacteriophages A3R and 676Z induce production of anti-phage IgG antibodies *in vivo* when applied both *per os* or i.p., but the onset of IgG production appears sooner in the i.p. route of administration. Major capsid protein contributes to the induction of humoral response in both models.

Acknowledgements
This work was supported by the National Science Centre in Poland grant UMO-2012/05/E/NZ6/03314 and by the Wroclaw Centre of Biotechnology, program The Leading National Research Centre (KNOW) for years 2014–2018.

Identification of prophages from genome sequence

Lichvariková Aneta[1]*, Belanová Veronika[1], Hyblová Michaela[2], Tomáš Szemes[2], Drahovská Hana[1], Turňa Ján[1,2]

[1] Comenius University, Slovak Republic, [2] Comenius University Science Park, Slovak Republic

*lichvarikova.aneta@gmail.com

Many bacterial genomes contain phage DNA integrated into the bacterial chromosome. Very often bacteria deposit multiple prophages that can constitute up to 20 % of total genome. Prophages are important genetic elements responsible for lateral gene transfer and interstrain genetic variability among several bacterial species. Prophage DNA has played an important role in evolution of pathogenic bacteria. They encode numerous virulence factors and pathogenicity islands and thereby play an important role in emergence of pathogen. In this work we focused on prophage identification from genome sequences of five *Streptococcus agalactiae* clinical strains. *S. agalactiae* represents opportunistic pathogen causing life-threatening infection in neonates and invasive diseases in adults. Prophages are not limited to pathogenic bacteria, some commensal and free-living bacteria show prominent prophage contribution to bacterial genome. The part of this work was also prophage identification from genome sequences of four LAB (Lactic Acid Bacteria) isolated from Slovak cheese bryndza which have potential as a starter culture during preparation of this ovine cheese.

Genome sequences were generated by whole genome sequencing using Illumina platform. The sequences were assembled with program CLC Bio (Qiagen) and annotated with RAST. Identification of prophage regions was done using web server Phast combined with analysis of annotated sequences. Five *S. agalactiae* strains studied in this work belong to serotype III, V and VII. We found out that each strain contains from 3 to 4 prophages with size from 15342 to 40850 base pairs. The prophage regions were compared with BLAST database, but no significant homology with known phages was found. Four LAB strains were classified as *Lactococcus lactis*, *Lactobacillus plantarum*, *Lactobacillus casei* and *Lactobacillus paraplantarum*. Each strain contains from 1 to 4 prophage regions with size ranging from 10415 to 69472 base pairs. The sequences of identified prophage regions were also analyzed using BLAST, but no significant homology was found. The prophage regions will be further analyzed. The information about prophage sequences of *S. agalactiae* strains as well as LAB will help to characterize the properties of this bacteria.

Funding

This work was supported by the Slovak Research and Development Agency under the contract No. APVV-14-0025 and UK/148/2017.

Preliminary studies of bacteriophages modified with peptides homing

Karolina Wojtyna*, Katarzyna Hodyra-Stefaniak, Dorota Lecion, Joanna Majewska, Zuzanna Kaźmierczak, Weronika Kęska, Anna Kłopot, Barbara Owczarek, Krystyna Dąbrowska, Andrzej Górski
Polish Academy of Sciences, Poland
*karolina.wojtyna@iitd.pan.wroc.pl

Peptides are widely known carriers that can manage targeting of a cargo in living organisms. Through recognizing vascular 'molecular addresses' they homing to organ of interest. In our study we focused on targeting peptides that homing to lungs. These peptides were displayed on bacteriophages able to kill bacteria, with the goal to adapt phages specifically to pulmonary bacterial infections. We hypothesize that phages displaying lungs-targeting peptides may accumulate in lungs, leading to enhancing antibacterial effect there. The second important is the influence of phages equipped with homing peptides on immunological system.

Using *in vivo* phage display method we successfully created phages T4-CGFECVRQCPERC and T4-CGSPGWVRC (phages T4 displaying peptides selectively binding to lungs vasculature). The T4 phage mutant T4Δ*hoc* served as a platform for phage display (Polish Collection of Microorganisms, IIET Wroclaw, Poland). The host for phage propagation was *Escherichia coli* expression strain B834 transformed with expression plasmids carrying the *hoc* gene in fusion with peptides CGFECVRQCPERC or CGSPGWVRC, respectively. The Hoc protein fused to peptides was incorporated into the surface of the T4Δ*hoc* phage during the phage assembly in the bacteria. Effectiveness of the phage display was confirmed by ELISA test. Additionally immunogenicity of constructed phages was examined in murine model and compared to the T4 phage. BALB/c mice were injected intraperitoneally with phages in dose 10^{10} pfu 3 times (day 0, 25 and 50). Blood samples were collected throughout the experiment (54 days) and evaluated for the presence of anti-phage IgG and IgM antibodies by ELISA. Bacterial transformation with modified plasmids resulted in effective expression of recombinant proteins, as confirmed by SDS-PAGE. Expression bacteria were effectively infected with the T4Δ*hoc* phage and targeted peptides were incorporated into phage capsids. Concentration of phages obtained after phage-display and purification (chromatography and dialysis) was 2 x 10^{12} pfu/ml for T4-CGFECVRQCPERC and 1.3 x 10^{12} pfu/ml for T4-CGSPGWVRC. As a result we observed efficient CGFECVRQCPERC-Hoc or CGSPGWVRC-Hoc fusions on the surface of phage particles, as confirmed with anti-Hoc antibodies in ELISA. There was no significant difference in IgM and IgG level between mice injected with engineered phages and T4 phage.

These observations demonstrate that short targeting peptides can be effectively incorporated into phage particles by phage display, as an N-terminal fusion to protein Hoc. Furthermore these peptides do not strengthen stimulation of anti-phage antibody production in mammals, which is important for potential therapeutic use. In the future studies bacteriophages T4-CGFECVRQCPERC and T4-CGSPGWVRC will be used to assess their homing properties. We propose these engineered phages for further applications in *in vivo* models.

Acknowledgements

This work was supported by National Science Centre, Poland, grant no. UMO 2014/13/N/NZ6/03985 and by the Wroclaw Centre of Biotechnology program The Leading National Research Centre (KNOW) for years 2014–2018.

New insights into A1, a multitasking pre-early protein of bacteriophage T5

Léo Zangelmi*, Madalena Renouard, Ombeline Rossier, Pascale Boulanger
Institute of Integrative Biology of the Cell, CNRS, University of Paris Sud, University Paris–Saclay, France
*leo.zangelmi@i2bc.paris-saclay.fr

Immediately after delivery of their genome into the host, lytic phages defeat bacterial defences and hijack host cell machineries to establish a favourable environment for their multiplication. The early-expressed genes governing host takeover are highly diverse from one phage to another and most of them have no assigned function. They thus represent a pool of novel genes whose products potentially subvert or disrupt bacterial cell vital functions for phage profit. So far, however, their characterization is still at an early stage.

Bacteriophage T5 uses a unique two-step mechanism to deliver its 121 kb DNA into its host Escherichia coli[1]. At the onset of infection only 8 % of the genome enters the cell before the transfer temporarily stops. During the pause, the genes encoded by this DNA portion are expressed and their products lead to the host chromosome degradation, shut-off of the host gene expression and inactivation of the host defence systems (restriction/methylation, DNA repair)[2]. After a few minutes, T5 DNA transfer resumes allowing expression of the T5 middle and late genes and further phage productive growth. This original mechanism of DNA delivery facilitates the identification and functional characterization of the early genes responsible for host takeover, as they are clustered on the genome.

Two early proteins of T5, A1 and A2, are required for resuming the DNA transfer and additionally A1 is essential for host DNA degradation. We have demonstrated that purified A1 has an exo- and endo-nuclease activity in vitro. Moreover, ectopic expression of A1 in E. coli is sufficient to observe host DNA degradation by fluorescence microscopy. Taken together, our results indicate that A1 might be the so far elusive phage factor responsible for the massive host DNA digestion observed after the first-step transfer. Using bioinformatics tools, site-directed mutagenesis and complementation assays, we have identified some amino acids that are essential for infection and nuclease activity. Our findings raise the intriguing question of how A1 nuclease activity is coupled to the completion of phage DNA transfer.

References

1. Lanni Y.T. (1968). First step-transfer deoxyribonucleic acid of bacteriophage T5. *Bacteriol Rev*. **32**: 227–42.
2. Davison J. (2015). Pre-early functions of bacteriophage T5 and its relatives. *Bacteriophage*. **5**(4): e1086500.

Molecular characterization of the activity and requirements of a novel and promiscuous bacteriophage integrase

Mohammed R. Mohaisen*, Alan J. McCarthy & Heather E. Allison
University of Liverpool, UK
*m.r.mohaisen@liv.ac.uk

Stx bacteriophages are responsible for the dissemination to and production of Shiga toxin genes (stx) in the Enterohaemorrhagic *E. coli* (EHEC). These toxigenic bacteriophage hosts can cause severe, life-threatening illness, and Shiga toxin (Stx) is responsible for the severe nature of EHEC infection. At the point of infection, the injected phage DNA can direct its integration into the bacterial chromosome becoming a prophage; the host cell is then known as a lysogen. Unusually, our model Stx phage, Φ24B, can integrate into at least four distinct sites within the *E. coli* genome that shared no easily identifiable recognition sequence pattern. The identification of what are actually required for phage and bacterial DNAs recombination has been tested using both an *in vitro* and *in situ* recombination assays. These assays enable easy manipulation of bacterial attachment site (attB) and phage attachment site (attP) sequences. The aim of our study is to fully characterize the requirements of this promiscuous integrase, carried by the Stx phage Φ24B (IntΦ24B), to drive integration.

So far, a number of successful assays have enabled us to identify the minimal necessary flanking sequences for all of four *attB* sites identified (21 bp and 49 bp from the right and lift the cross over region) and *attP* site (200 bp each side). Furthermore, we identified that Φ24B integrase does not need Integration Host Factor (IHF) to drive integration. Finally, as this integrase can integrate the phage genome inside more than four different bacterial attachment sites *attBs*, four of these *attBs* were identified, sequenced and cloned in different compatible plasmids to be transformed to one cell, and the frequency of each recombination was tested by means of qPCR.

Genomes mining and prediction of Streptococcus phage receptor binding proteins

Jessica Jakabova[1], Lenka Tisakova[1], Andrej Godany[1,], Barbora Vidova[2*]
[1]University of Ss. Cyril and Methodius, Slovakia, [2]Slovak Academy of Sciences, Slovak Republic
*barbora.vidova@savba.sk

Phage receptor binding proteins specifically allows bacteriophages to recognize their target bacteria. Due to their high specificity these proteins are characterized by great variability in structure and their analysis, as well as characterization of interactions with host receptors is necessary for the success application. Here we have analysed the tail regions of selected bacteriophages infecting the genus *Streptococcus* focusing on binding domains of receptor binding proteins (RBPs) located within these regions. The work comprised 78 streptococcal phage genomes, among which phylogenetic relationship were analyzed. From this set 30 were subsequently selected due to their content of receptor-like ORFs with binding domain functions. Four different types of ORFs were identified, encoding receptors formed by four domain families, namely by collagen triple helix repeat, prophage endopeptidase tail, cis(Z)-isoprenyl diphosphate synthases, and chitin-binding domains. Tertiary structures of domains were predicted, along with structural models of potentially suitable ligands, and ligand binding sites with predicted role in the interaction with the host cell.

The obtained results extend the knowledge about presence of receptor binding proteins in Streptococcal genomes, their types, sequence organization, and interaction with components of the cell wall, thus this work opens new perspectives on their use as in phage therapy, as well as in identification of the host bacteria.

Acknowledgements

This work was supported by VEGA grant no. 2/0099/16 “Analysis of the Receptor Binding Proteins of Streptococcal, Staphylococcal, Streptomycetal and Enterococcal phages” from the Scientific Grant Agency of the Ministry of Education, Sciences, Research and Sports of the Slovak Republic, and Slovak Academy of Sciences.

Iron-Virus interactions in the oceans

C. Bonnain*, K.N. Buck, M. Breitbart
University of South Florida, United States
*cbonnain@mail.usf.edu

Iron is an essential nutrient in the oceans, with the sub-nanomolar concentrations found in open ocean surface waters often insufficient for supporting biological activity. More than 99.9 % of dissolved iron is bound to organic ligands, yet identifying the sources of these ligands in seawater remains a major challenge. A significant portion of iron-binding ligands fall into the colloidal fraction, which is operationally defined as the fraction collected between a 0.02 µm and a 0.45 µm filter. Among the organic ligands in this fraction persists an extremely abundant biological candidate: viruses. On average there are 10^7 viruses per milliliter of seawater, most of which are phages (viruses that infect bacteria).

The impact of viruses on ocean biogeochemistry is often evoked purely through the act of lysing hosts and very few studies have considered the geochemical potential of the viral particles themselves. Recent work in non-marine model systems has revealed the presence of iron atoms within the structure of diverse phages infecting *Escherichia coli*. Combined with the small size and sheer abundance of phages in the oceans, the inclusion of iron in phage structures would translate into a major factor for cycling of this important trace metal. In addition, iron is so critical for growth that bacteria have evolved multiple uptake systems for assimilating iron, such as siderophores.

Certain outer membrane proteins serve a dual function in siderophore uptake and as a phage receptor, suggesting that some of the strategies utilized for iron acquisition make bacteria vulnerable to phage infection. Given the constant arms race between bacteria and phages to develop resistance and counter-resistance, respectively, it is not surprising that phage would have evolved to utilize critical regions of surface-exposed proteins which are indispensable for bacterial growth as receptors. The research presented here explores the potential of marine phages to serve as iron-binding ligands and discusses the implications for both trace metal biogeochemistry and marine phage-host interactions.

Characterization of *Bdellovibrio bacteriovorus* phage

Kema Malki*, Bentley Fane, Mya Breitbart
University of South Florida, United States
*kema@mail.usf.edu

Bdellovibrio are small, parasitic bacteria that are best known for their ability to attack and lyse gram-negative bacterial cells. These δ-Proteobacteria were originally isolated from soil in the 1960s during a search for bacteriophage (phage). Since then, different strains of Bdellovibrio with varying host ranges have been discovered from diverse environments including freshwater, seawater, soil, and sewage. Though originally thought to be obligate parasites, strains of host-independent Bdellovibrio have also been isolated and cultured in laboratory settings. Due to their capability to lyse bacteria, Bdellovibrio have important ecological implications and have even been proposed as potential antimicrobial agents for human health applications.

The purpose of this study is to expand our knowledge of phage capable of infecting *Bdellovibrio bacteriovorus*. Several studies in the early 1970s isolated Bdellovibrio phage and described their morphology and infection parameters. However, to date, genomic information is only available for two Bdellovibrio phage, the small ssDNA phage ΦMH2K and the dwarf myovirus Φ1402, which is the smallest autonomous myovirus. These phage genomes suggest that Bdellovibrio phage may represent a large reservoir of unexplored diversity, particularly regarding phage with reduced genome sizes. In this study, phage capable of creating plaques on the host-independent, *Bdellovibrio bacteriovorus* strain HI 109 were isolated from sewage samples, plaque purified, and are currently being characterized based on their morphology and genome sequences. The availability of well-characterized Bdellovibrio phage systems in the lab represents a critical step towards future work examining the net ecological impact of Bdellovibrio phage on environmental bacterial communities.

Controlling *Acinetobacter baumannii* infections in abiotic and biotic surfaces with bacteriophages

Ana Rita Costa*, Nico Konstantinides, Rodrigo Monteiro, Luís Melo, Joana Azeredo
University of Minho, Portugal
*anaritamc@deb.uminho.pt

Acinetobacter baumannii has emerged as a serious nosocomial pathogen, particularly due to the alarming development of resistance to most classes of antibiotics. It is therefore important to explore alternative antimicrobials, such as bacteriophages, to fight these infections. So, the aim of this work was to isolate lytic phages against *A. baumannii*, and to evaluate their efficacy on the control of this bacteria in abiotic and biotic surfaces.

Thirteen phages were isolated from wastewater samples and characterized, generally demonstrating a limited host range (average 13 %, min. 4 %, max. 20 %, n= 25), and belonging to the *Myoviridae* and *Podoviridae* families (as determined by transmission electron microscopy). Phage activity against 24h biofilms in 96-well plates and 2h and 24h infections in human alveolar epithelial cells (A548 cells) was determined, with phages being most active after 6h, and bacterial resistance emerging after 24h in most cases. Results varied among phages, but it was possible to reduce the bacterial loads by 2-3 logs, a reduction higher than that achieved by most antibiotics.

To summarize, phages demonstrated to be promising antimicrobials to control *A. baumannii* infections in both biotic and abiotic surfaces. Further improvements may be achieved using combinations of phages or phages and antibiotics.

Synergistic antimicrobial interaction between honey and bacteriophage against *E. coli* biofilms

Henrique Ribeiro, Ana Oliveira, Ana C. Silva, Maria D. Silva, Jéssica C. Sousa, Célia F. Rodrigues, Luís D.R. Melo, Ana F. Henriques, Sanna Sillankorva*
University of Minho, Portugal
*s.sillankorva@deb.uminho.pt

Chronic wounds afford a hostile environment of injured tissues that allow bacterial proliferation and further wound colonization. The most common colonizer of infected wounds is *Escherichia coli* that is a prolific biofilm former. Living in biofilm communities, cells are protected, becoming more difficult to control and eradicate, and less susceptible to antibiotic therapy. This work presents insights into the proceedings triggering *E. coli* biofilm control with phage, honey and their combination, achieved through standard antimicrobial activity assays and flow cytometry studies and further visual insights sought by SEM and TEM microscopy.

A Portuguese honey (PF2) and an *E. coli* specific phage (EC3a), possessing depolymerase activity, were tested against 24h- and 48h-old biofilms. Synergic effects were perceived in phage-honey experiments. Combined treatments prompted similar phenomena in biofilm cells, visualized by electron microscopy, as the individual therapy. Honey caused minor membrane perturbations to complete collapse and consequent discharge of cytoplasmic content, and phage completely destroyed cells leaving only vesicle-like structures and debris. Our experiments show that the addition of phage to low honey concentrations is advantageous.

Portuguese honey possesses excellent anti-biofilm activity and may be a potential alternative therapeutic agent in biofilm-related wound infection. Furthermore, the phage-honey combination approach is more valuable than phage therapy alone, since the antiviral effect of honey limits the emergence of phage resistant phenotypes

Genomic analysis of *Acinetobacter baumannii* prophages reveals remarkable diversity and suggests significant impact on bacterial virulence and fitness

Rodrigo Monteiro, Ana Rita Costa, Joana Azeredo*
University of Minho, Portugal
*jazeredo@deb.uminho.pt

Bacterial genomics has revealed substantial amounts of prophage DNA in bacterial genomes. This integrated viral DNA has been shown to play important roles in the evolution of bacterial pathogenicity. *Acinetobacter baumannii* has shown a fast progression as a nosocomial multi-resistant pathogen in recent years, and is now considered one of the most dangerous microorganisms in hospital environments. The role of prophages in the evolution of *A. baumannii* pathogenicity has not yet been explored. In this context, we aimed at evaluating the impact of prophages on *A. baumannii* genomic diversity and pathogenicity.

Approximately 959 strains were analyzed for the presence of intact and defective prophages using PHAST. A total of 6691 prophages were detected, with all strains having at least one prophage and 83.4 % encoding intact prophages. A subset of 184 prophages (from 134 strains) were analyzed in more detail. Prophages were classified by comparing of specific structural proteins to those of previously classified phages using BLASTp. Among the prophages possible to classify, all belonged to the *Caudovirales* order, with higher prevalence of *Siphoviridae* (39.67 %), followed by *Myoviridae* (19.57 %) and *Podoviridae* (8.7 %) families. The prophages sequences were aligned using MAFFT, and a distance matrix and a phylogenetic tree were constructed to evaluate similarities. A high diversity was found among the prophages, which may contribute to the diversity of *A. baumannii*, since some strains differ only on the integrated prophages. Furthermore, numerous potential virulence factors encoded by the prophages were detected, implicated in *A. baumannii* pathogenicity, namely antibiotic resistance, toxins, host interaction, survival and fitness.

Overall, our results demonstrate a high prevalence of prophages in *A. baumannii*. The amount and diversity of potential virulence factors encoded by the identified prophages point towards a significant contribution of these mobile elements for the dissemination and evolution of pathogenicity in this bacterial species.

Development of a phage cocktail to control *Proteus mirabilis* CAUTIs

Luís D.R. Melo[1]*, Patrícia Veiga[1], Nuno Cerca[1], Andrew M. Kropinski[2, 3], Carina Almeida[1], Joana Azeredo[1], Sanna Sillankorva[1]

[1]University of Minho, Portugal, [2]Public Health Agency of Canada, Canada, [3]University of Guelph, Canada

*lmelo@deb.uminho.pt

Proteus mirabilis is an enterobacterium that causes catheter-associated urinary tract infections (CAUTIs) due to its ability to form crystalline biofilms on the surfaces. These CAUTIs are very difficult to treat, since the biofilm structures are extremely tolerant to high concentrations of antibiotics. Bacteriophages (phages) have been used widely to control and prevent a diversity of bacterial species, however a limited number of phages for *P. mirabilis* have been isolated and studied. Here we report the isolation of two novel virulent phages, the myovirus vB_PmiM_5460 and the podovirus vB_PmiP_5461 able to target respectively 57 % and 100 % of all *Proteus* strains tested in this study. Both phages have been characterized thoroughly and sequencing data revealed no traces of genes associated with lysogeny. To further evaluate the phages ability to prevent catheter colonization by *Proteus*, phages adherence to silicon surfaces was assessed. Both phages were able to adhere, but the extent of adhesion was found to be phage dependent. Further tests in phage-coated catheters using a dynamic biofilm model simulating CAUTIs, have shown a 90 % significant reduction of *P. mirabilis* biofilm formation up to 168 h of catheterization. These results highlight the potential usefulness of the two isolated phages for the prevention of surface colonization by this bacterium.

References

1. Armbruster C.E., Mobley, H.L. (2012) Merging mythology and morphology: the multifaceted lifestyle of *Proteus mirabilis*. *Nat Rev Microbiol* **10**: 743-754.
2. Carson, L., Gorman, S.P., Gilmore, B.F. (2010) The use of lytic bacteriophages in the prevention and eradication of biofilms of *Proteus mirabilis* and *Escherichia coli*. *FEMS Immunol Med Microbiol* **59**: 447-455.
3. Getliffe, K., Newton, T. (2006) Catheter-associated urinary tract infection in primary and community health care. *Age Ageing* **35**: 477-481.

Bacteriophage depolymerases – novel polysaccharide degrading enzymes

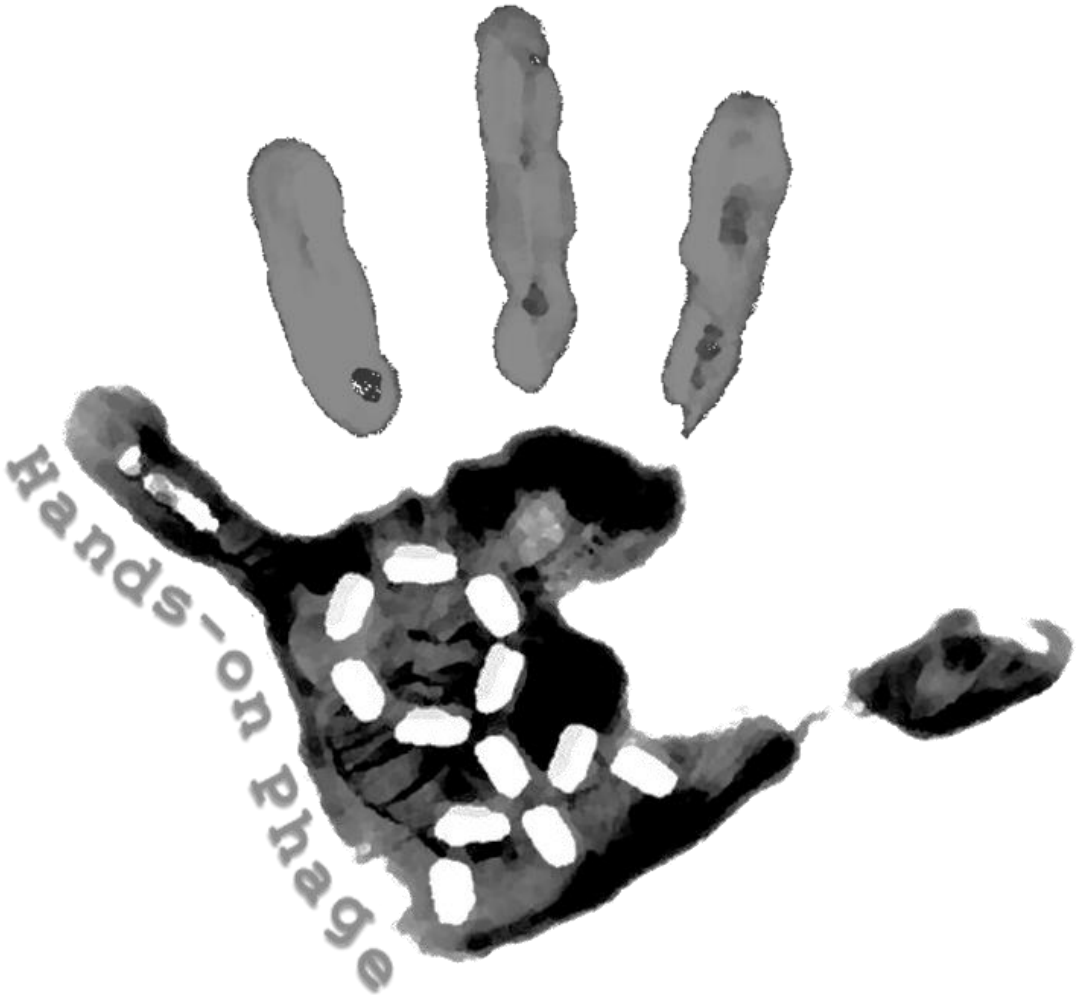
Hugo Oliveira*, Diana P. Pires, Luís D. R. Melo, Leon Kluskens, Sanna Sillankorva, Joana Azeredo
University of Minho, Portugal
*hugooliveira@deb.uminho.pt

In nature, biofilms are the most common lifestyle of bacteria and are difficult to eradicate, partially due to the extracellular polymeric substances content of the slime in which biofilms are embedded, which act as a primary defence against disinfection. (Bacterio)phages are viruses that specifically infect bacteria and can represent an important strategy for biofilm control. These viruses have evolved specialized enzymes, called depolymerases, to degrade polymers present in the bacterial surface and slime (e.g. capsular and structural polysaccharides) to facilitate access to their hosts.

We performed an *in silico* analysis of all available phage genomes infecting different bacteria and found 160 putative depolymerises with specialized activity (e.g. hyaluronidases, alginate and pectate lyases). This illustrates how well phages are equipped to degrade a diverse range of biofilm-associated polymers.

We cloned and recombinantly expressed an *Acinetobacter* phage depolymerise and demonstrated its activity by the spot-on-lawn method. To further characterize the enzyme activity, the bacterial host genome was sequenced and a cluster (24.6 kb) of genes responsible for the capsular polysaccharide biosynthesis was identified. Using DNA recombination, mutations in either of the two transcribing strands were introduced to generate capsular polysaccharides deficient mutants. Results demonstrate that the depolymerase specifically degrades *Acinetobacter* capsular polysaccharides.

Overall, *in silico* and experimental results suggest that phage represent a source of enzymes to degrade polymeric substances presence in bacterial slime, which can be further exploited for biofilm control.



PRACTICAL SESSIONS

3rd International hands-on PHAGE BIOTECHNOLOGY course

JUNE 19-23, 2017 | BRAGA-PORTUGAL

PRACTICAL SESSION P1

Bacteriophage isolation, production and titration

Diana Priscila Pires*, Carla Carvalho, Ana Oliveira, Catarina Milho, Ergün Aktürk, Henrique Ribeiro, Maria Daniela Silva, Rodrigo Monteiro

*priscilapires@deb.uminho.pt

1. INTRODUCTION

Bacteriophage isolation: Bacteriophages (phages) can be found in places where their hosts exist. The total population of phages is now estimated to be 10^8 species and 10^{32} particles in the biosphere, making them the most abundant biological entities on this planet[1-3]. In this work we propose the isolation of different phages[4-10], which can be found in places where their corresponding hosts are present. For that, samples of solid and liquid sources will be used (e.g. sewage from wastewater, sludge/soil). The method used in our practical session increases the likelihood of detecting and isolating phage present in very low numbers by using a selective phage-enrichment technique[11,12]. Field samples will be homogenized in culture medium, and incubated with potential host bacterial strains to propagate the phage. Then, the lytic activity of the enriched supernatant will be tested against each of the bacterial strains used in the enrichment step. Soft agar will be used to allow phages to easily diffuse through the medium, resulting in a more consistent clear area. During incubation, the uninfected bacteria multiply to form a confluent lawn over the surface of the plate, while infected bacteria bursts after a short time post-infection. The latter event results in a lysis zone indicative of phage presence. To discriminate different phages, a positive sample will be serial diluted and plated. Discrete phage plaques will be observed, representing bacterial lysis caused by a single phage. Plaque characteristics are related to the type of phage, bacterial host characteristics as well as to other physical and chemical characteristics of the system in which the phage was produced[13-15]. Different phage plaques, if observed, will be segregated.

Bacteriophage production: Phage production is essential to obtain a sufficient amount of phages for subsequent characterization and assessment of their potential use in different applications. Three different methods will be presented herein for phage production that can serve the majority of purposes in phage investigation.

Bacteriophage titration: After production, phage titration is required to determine the amount of phage particles that are present in a given sample. We describe herein the drop plaque and the double agar overlay methods for this purpose.

Bacteriophage purification: After production, purification is a vital step to ensure a pure and debris-free phage suspension that can be used for several applications. Due to the length of this procedure, a detailed protocol for polyethyleneglycol (PEG 8000) precipitation and CsCl gradient ultracentrifugation is given as supporting material (P1 Supplementary Protocol).

2. MATERIALS

Prepare all solutions using distilled water. The media used in the procedures described herein is Tryptic Soy Broth (TSB) (see **note 1**) but alternative media can be used, depending on the bacterial species. Sterilize (autoclave at 121 °C, 1 bar, for 15 min) all the solutions and material and store at room temperature unless indicated otherwise.

2.1. Bacteriophage enrichment from collected samples

- Samples for phage isolation:
  - solid samples (e.g. soil/ sludge): place in jars
  - liquid samples (e.g. sewage): place in 1 L bottles
- 50 mL centrifuge tubes
- Double strength TSB (2× TSB) broth: Add 48 g of TSB (see **note 1**) to 800 mL of distilled water
- TSB broth: Add 24 g of TSB to 800 mL of distilled water (see **note 1**)
- Overnight grown bacterial hosts
- 100 mL and 500 mL bottles
- 500 mL Erlenmeyer flasks
- 0.22 µm PES filters
- Syringes
- Saline solution (0.9 % (w/v) NaCl): Add 3.6 g of NaCl to 400 mL of distilled water

2.2. Preparation of bacterial lawns by pour-plate technique

- TSA agar plates (1.2 % (w/v) agar): Add 24 g of TSB and 9.6 g of agar to 800 mL of distilled water. After autoclaving pour on petri dishes
- Molten Top-Agar (MTA_TSB) (0.6 % (w/v) agar): Add 15 g of TSB and 3 g of agar to 500 mL distilled water. After autoclaving store accordingly (see **notes 2 and 3**)
- Overnight grown bacterial hosts
- 15 mL test tubes

2.3. Bacteriophage isolation

- Overnight grown bacterial hosts
- MTA _TSB (47 °C) (see **notes 2 and 3**)
- TSA plates
- Sterile paper strips (approximately 1 cm × 5 cm)
- Sterile toothpicks

2.4. Bacteriophage production

- Overnight grown bacterial host
- TSA plates or 75-150 cm^2 tissue culture flasks containing TSA
- MTA_TSB (47 °C) (see **notes 2 and 3**)
- SM (saline magnesium) buffer: Prepare 1 M Tris-HCl buffer (pH 7.5) in a 100 mL bottle. Weigh 6.06 g

of Tris-Base, add 50 mL of water and adjust the pH to 7.5 with HCl. Then, to a 1 L bottle, add 5.8 g of NaCl, 2 g of $MgSO_4.7H_2O$ and 50 mL of the prepared 1 M Tris-HCl (pH 7.5), and make up to 1 L with water (see **note 4**)

- 50 mL centrifuge tubes
- 250 mL Erlenmeyer flasks
- 50, 100, and 200 mL bottles
- Sterile paper strips (see 2.3)
- 0.22 µm PES filters
- Syringes
- Sterile toothpicks
- Chloroform

2.5. Bacteriophage titration

- TSA plates
- MTA_TSB (47 °C) (see **notes 2 and 3**)
- SM buffer (see **note 4**)
- 1.5 mL microcentrifuge tubes
- 15 mL centrifuge tubes

3. METHODS

Carry out all procedures at room temperature and under aseptic conditions, using flame or inside a laminar flow chamber.

Only non-lysogenic strains should be used in the enrichment procedures, to avoid false positives. A lysogen is a bacterium that contains an inducible prophage (that is capable of infecting other hosts) and is detected through the use of an inducing agent:
"The most sensitive method is thus induction by mitomycin C or UV light (or a combination of both) followed by the spot test in combination with electron microscopic examination"
Ackermann and DuBow

More information in Ackermann and DuBow 1987, Viruses of prokaryotes. V. 2, General properties of bacteriophages. CRC Press.

3.1. Bacteriophage enrichment

1. Put 50 mL of the liquid samples in 250 mL sterile Erlenmeyer flasks; For solid samples, add 50 mL of saline solution and 10 g of solid sample to 250 mL Erlenmeyer flasks.
2. Add 50 µl of overnight grown bacterial suspensions (at least 5 different non-lysogenic strains) (see **note 5**) and 50 mL of 2× TSB to the Erlenmeyer flask containing the samples.
3. Incubate at 37 °C, under agitation (120-180 rpm), during 24 to 48 h (see **note 6**).

4. Pour the enriched sample in 50 mL centrifuge tubes and centrifuge (9,000 ×*g*, 4 °C for 10 min).
5. Collect and filter (0.22 µm) the supernatant to sterile 50 mL centrifuge tubes.

3.2. Preparation of bacterial lawns by pour plate technique

1. Add 100 µL of overnight grown bacterial suspension and 3-5 mL of MTA_TSB (47 °C) to a 15 mL test tube and tap gently.
2. Pour onto an agar plate with TSA and swirl gently.
3. Let the plates dry for 1-2 min.

3.3. Bacteriophage isolation

3.3.1. Spot test verification of the enriched samples

1. Add 10 µL of the filtered sample (step 3.1.5) on a bacterial lawn (prepared as described in 3.2) of the strain(s) used in the enrichment (see **note 7**).
2. Let the plate stand until completely dried.
3. Incubate the plate overnight at the proper growth temperature.
4. Check for clear and turbid lysis zones indicative of the presence of phages (lysis zones can also be due to other factors like the presence of bacteriocines; to ensure that the observed phenomena is due to phage activity, you need to go to step 3.3.2).

3.3.2. Bacteriophage plaque isolation

Positive results from step 3.3.1 need to be investigated for the presence and further isolation of different phages in the enriched samples using the procedure as follows:

1. Wet the tip of a sterile paper strip (see **note 8**) in the phage suspension obtained in 3.1.5.
2. Streak once on a Petri dish containing a bacterial lawn (prepared as described in 3.2) of the strain used in the enrichment step (Fig. P1.1A).
3. Streak downwards, changing the paper strip after every streak, making certain that the paper strip touches the previous streak (Fig. P1.1B).
4. Incubate the plate overnight at optimum temperature for bacterial growth.
5. Analyse the phage plaque morphologies to check for differences in size, presence of halo, turbidity, etc (examples of plaques with varying morphology in Fig. P1.1C).
6. Pick different plaques with a toothpick and stick the toothpick several times (in a line), in an agar plate with a bacterial lawn prepared as described in 3.2. (Fig. P1.1D).
7. Use sterile paper strips to streak the phages as described above (3.3.2.3) (Fig. P1.1E).
8. Incubate the plates with different plaque morphologies overnight at the proper temperature and repeat steps 6-7 until all phage plaques are uniform (Fig. P1.1F) (see **note 9**).
9. Store at 4 °C until needed (phage stock).

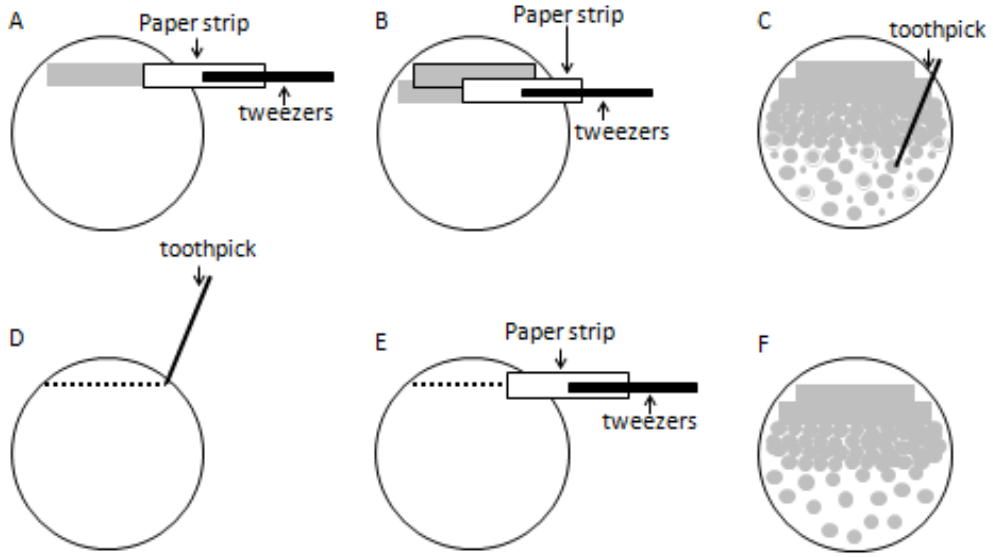


Fig. P1.1. Isolation of bacteriophages. (**A**) Initial streak; (**B**) Streaking downwards on a bacterial lawn; (**C**) Isolated phage plaques and picking a single plaque with a toothpick; (**D**) Making a puncture line with a toothpick; (**E**) Streaking with paper strips; (**F**) Morphologically identical plaques.

3.4. Bacteriophage production

3.4.1. Bacteriophage production from single phage plaques using the plate lysis and elution procedure

1. Pick with a toothpick a phage plaque and stick it on 1 to 20 agar plate(s) containing a bacterial lawn prepared as described previously (3.2.).
2. Without changing the paper strip, pass it through the punctures made, and spread evenly to ensure maximum coverage (maximum replication) of the phages.
3. Incubate overnight at the proper temperature.
4. Add 3-5 mL of SM buffer to each plate used.
5. Incubate with agitation (50- 90 rpm) at 4 °C during 5-18 h.
6. Collect the SM buffer with the eluted phages to 50 mL centrifuge tubes (see **note 10**) and discard the agar plate.
7. Add chloroform to a final concentration of 10 % (v/v).
8. Centrifuge the solution (9,000 ×*g*, 4 °C, and 10 min) to remove all bacteria.
9. Collect and filter (0.22 µm) the supernatant to 100 mL bottles.
10. Store at 4 °C until needed.

3.4.2. Bacteriophage production using the soft-agar overlay technique

1. Add 1 mL of the bacterial culture grown overnight and 1 mL of diluted phage (approximate titre 1×10^5 plaque forming units (PFU) per mL) in tissue culture flask(s) (75 cm^2) containing an agar layer of TSA, and mix gently. Alternatively, production can be made in TSA plates: add 100 µl of the bacterial culture grown overnight and 100 µL of diluted phage in TSA plates, and mix gently.
2. Incubate for 10 min at the proper temperature to allow phages to adsorb to the host bacterium.
3. Add 25 mL of MTA_TSB (47 °C) (3-5 mL when production is on TSA plates) and hold the tissue culture flask(s) still until the agar has hardened/solidified.

4. Incubate overnight, without inverting, at the proper temperature.
5. Add 25 mL of SM buffer (3-5 mL when production is on TSA plates) to the tissue culture flask(s) (75 cm^2) and incubate overnight at 4 °C.
6. Transfer the SM buffer with the eluted phages to 50 mL centrifuge tubes (see **note 10**).
7. Add chloroform to a final concentration of 10 % (v/v).
8. Centrifuge the solution (9,000 ×*g*, 4 °C, and 10 min) to remove all bacteria.
9. Carefully collect the supernatant, filter (0.22 µm) and transfer to sterile 50 mL (1 tissue culture flask used) or 200 mL (5 tissue culture flasks used) bottles.
10. Store at 4 °C until further use.

3.4.3. Bacteriophage production using a bacterial culture

1. Grow bacteria in 100 mL Erlenmeyer flasks containing 25 mL of sterile TSB, until cells reach mid-exponential phase (OD_{600} = 0.4).
2. Dilute the phage stock solution to have a concentration of approximately 1×10^5 PFU/mL.
3. Add 1-5 mL of the diluted phage and 25 mL of the bacterial culture (from step 1) to a 250 mL Erlenmeyer flask.
4. Incubate at proper temperature and under agitation (150-200 rpm).
5. When the culture turbidity decreases (4-6 h), transfer to 50 mL centrifuge tubes.
6. Add chloroform to a final concentration of 10 % (v/v).
7. Collect, filter (0.22 µm) and transfer the supernatant to 100 mL bottles.
8. Store at 4 °C until needed.

3.5. Bacteriophage titration

First, prepare ten-fold serial dilutions in SM buffer of the phage stock solutions (add 100 µL of phage solution and 900 µL of SM buffer to 1.5 mL tubes).

3.5.1. Enumeration using the drop plaque assay

1. Add 100 µL of bacterial culture grown overnight, and 3-5 mL of MTA_TSB (47˚C) to a TSA plate and gently mix.
2. Let the plates dry for 10 min.
3. Add a drop of 10 µL of the dilution mixture onto a TSA plate, and tilt the plates at 45°.
4. Let the plates stand still until the drops have completely dried.
5. Incubate overnight at the proper growth temperature.
6. Count the plaques formed in the drop of the dilution with 3-30 phage plaques (Fig. P1.2).
7. Calculate the phage titre of duplicated preparations using Equation P1.1.

Equation P1.1:

$$Bacteriophage\ titer\ (PFU\ per\ mL) = \frac{Nr.of\ bacteriophage\ plaques\ formed\ \times Dilution\ factor}{Volume\ of\ bacteriophage\ sample\ (mL)}$$

3.5.2. Enumeration by double agar overlay

1. Using the prepared serial dilutions, add 100 µL of phage solution, 100 µL of bacterial culture grown

overnight, and 3-5 mL of MTA_ TSB (47 °C) to a TSA plate and gently mix.

2. Let the plates dry for 10 min.
3. Incubate overnight under optimal conditions.
4. Count the phage plaques in the dilution which resulted in 20-200 plaques (Fig. P1.2).
5. Determine the titre of duplicated preparations according to Equation P1.1.

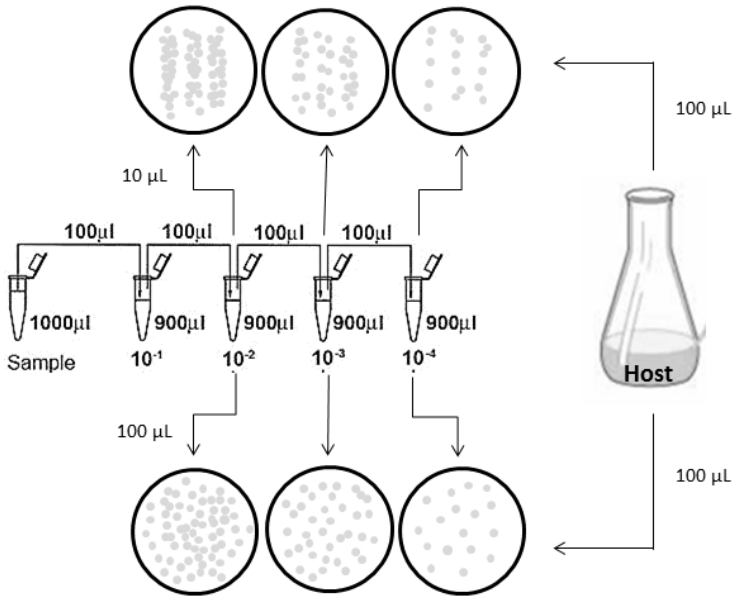


Fig. P1.2. Phage enumeration. Phages can be enumerated using the drop plaque assay (upper panel) and by double agar overlay (lower panel).

4. NOTES

1. TSB medium is a rich medium that is commonly used to culture members of the Enterobacteriaceae as well as for coliphage plaque assays. TSB is commercially available otherwise prepare as follows: to 1 L of distilled water add 17.0 g of Enzymatic Digest of Casein (Tryptone); 3.0 g of Enzymatic Digest of Soybean Meal (Soytone); 5.0 g of NaCl; 2.5 g of dipotassium phosphate (K_2HPO_4); and 2.5 g of Dextrose.
2. MTA_TSB is generally prepared with 0.6 % (w/v) of agar; however agar percentages between 0.4 % and 0.7 % can be used. Alternatively, MTA can be prepared with agarose instead of agar.
3. MTA_TSB can be stored at 47 °C if used within 1 day or at 4-21 °C for longer periods. Solid MTA can be melted using a water bath or a microwave oven.
4. Optional: 2 % (w/v) of gelatin can be added to SM buffer. Gelatin is known to preserve phages and thus can be used in the later steps of phage purification.
5. A loopful of freshly grown host bacterium can be picked from agar plates and suspended in saline solution (0.9 % (w/v) NaCl) and used instead of overnight grown bacterial suspension.
6. The culture media and the temperature depends on the bacterial species used.
7. Several different phage enrichment samples, from different sources and origins, can be spotted on one bacterial lawn.
8. The paper strip has the same functionality for streaking phages as the inoculating loops have for streaking colonies.
9. Note that it is possible that one single phage plaque consists of plaques with different sizes. This can be

confirmed by repeating the isolation process for each different plaque and observe if the same result is obtained.

10. The MTA_TSB layer can also be collected.

5. REFERENCES

1. Chibani-Chennoufi S., Bruttin A., Dillmann M-L., Brussow H. (2004). Phage-Host Interaction: An Ecological Perspective. *J Bacteriol* 3677–3686.
2. Hanlon G. W. (2007). Bacteriophages: an appraisal of their role in the treatment of bacterial infections. *Int Antimicrob Agents* **30**(2): 118–128.
3. Abedon S. T. (2006). The Bacteriophages. 2nd ed: Oxford University Press.
4. Oliveira A, Sillankorva, S, Quinta R, Henriques A, Sereno R, Azeredo J. (2009). Isolation and characterization of bacteriophages for avian pathogenic *E. coli* strains. *J Appl Microbiol* **106**(6): 1919-27.
5. Gutiérrez D, Martínez B, Rodríguez A, García P. (2010) Isolation and characterization of bacteriophages infecting *Staphylococcus epidermidis*. *Curr Microbiol* **61**(6): 601-8.
6. Hatfull G.F., Cresawn S.G., Hendrix R.W. (2008). Comparative genomics of the mycobacteriophages: insights into bacteriophage evolution. *Res Microbiol* **159**(5): 332-9.
7. Pires D.P.P., Sillankorva S., Faustino A., Azeredo J. (2011). Use of newly isolated phages for control of *Pseudomonas aeruginosa* PAO1 and ATCC10145 biofilms. *Res Microbiol* **162**: 798-806.
8. Sillankorva S., Kropinski A.M., Azeredo J. (2012). Genome sequence of the broad-host range *Pseudomonas* phage phi-S1. *J Virol* **86**(18): 102-39.
9. Adriaenssens E.M., Mattheus W., Cornelissen A., Shaburova O., Krylov V.N., Kropinski A.M., Lavigne R. (2012). Complete genome sequence of the giant *Pseudomonas* phage Lu11. *J Virol* **86**(11): 6369-70.
10. Sillankorva S., Neubauer P., Azeredo J. (2008). Isolation and characterization of a T7-like lytic phage for *Pseudomonas fluorescens*. *BMC Biotechnol* **8**: 80.
11. Sambrook J. and Russell D.W. (2001). Molecular cloning - a laboratory manual. Third ed: Cold Spring Harbor Laboratory.
12. Santos S.B., Carvalho C.M., Sillankorva S., Nicolau A., Ferreira E.C., Azeredo J. (2009). The use of antibiotics to improve phage detection and enumeration by the double-layer agar technique. *BMC Microbiol* **23**(9): 148.
13. d'Herelle F. (1917). Sur un microbe invisible anatagoniste des bacilles dysenterique. C. R. Acad. Sci. **165**: 373-375.
14. Carlson K. Working with bacteriophages: common techniques and methodological approaches. In: Kutter E, Sulakvelidze A, editors. Bacteriophages: Biology and Application. Boca Raton, FL: CRC Press; (2004). pp. 437–494.
15. Jones D. and Krueger A. P. (1951) A Rapid Slide Plaque Technic for Bacteriophage Assay. *J. Gen. Physiol.* **34**: 347-357.

P1 SUPPLEMENTARY PROTOCOL

S1. Phage purification

Phages can be purified from crude bacterial lysates by several methods[1]. The addition of polyethylene glycol (PEG), for example, is a mild and fast procedure allowing a 100-fold phage concentration after low speed centrifugation with negligible loss of infectivity. If desired, phages can be further purified by isopycnic centrifugation through Cesium chloride (CsCl) gradients, which yields phages of the highest purity. The densities of the different CsCl layers are chosen so that the density range encompasses the proper buoyant density of the phage. If this latter value is unknown or if it cannot be estimated from the phage physical characteristics, it may be necessary to test several CsCl layer density patterns to optimize the purification.

S1.1. MATERIALS

S1.1.1. PEG purification

- SM buffer (see note 4 from P1)
- Chloroform
- NaCl, high purity grade
- PEG 8,000 (MW 5,000 - 7,000 g/mol) powder
- 0.22 µm PES filters
- 50 mL centrifuge tubes

S1.1.2. CsCl purification

- CsCl, ultrapure grade
- SM buffer
- Chloroform
- Ultracentrifuge tubes (see **note 1**)
- Syringes and 18-22 gauge hypodermic needles
- Dialysis tubing: MWCO 12,000-14,000
- Serological pipettes

S1.2. METHODS

S1.2.1. PEG purification

1. After transferring the SM buffer with the eluted phages to 50 mL centrifuge tubes (step 3.4.2 in Practical Session P1), dissolve solid NaCl into the phage suspension to a final concentration of 0.5 M and incubate at 4 °C for 1 h.

2. Remove the denser bacterial debris by centrifuging the suspension at 6,000-8,000 ×*g* for 10 min at 4 °C.

3. Transfer the phage-containing supernatant into a clean flask and dissolve PEG 8,000 to a final concentration of 8 %-10 % (w/v) at 4 °C, by brief stirring, and let it stand at 4 °C for at least overnight to precipitate the phage particles.
4. Sediment the precipitated phage at 10,000 ×*g* for 15 min at 4 °C and carefully discard the supernatant.
5. Place the centrifuge bottles backwards and let the remaining fluid drain away from the pellet for 5 min.
6. Gently suspend the pellet in SM buffer (1-2 mL per 100 mL of initial supernatant). Since phage particles may be damaged by vortexing or vigorous pipetting, it is recommended to leave it overnight at 4 °C to soften the pellet, which facilitates the suspension.
7. Separate phage particles from co-precipitated bacterial debris by low-speed centrifugation for 10 min at 5,000 ×*g*, at 4 °C.
8. If no further purification is needed, the residual PEG and bacterial debris can be removed by gentle extraction for 1 min with an equal volume of chloroform. The phage containing aqueous phase is separated from the white organic phase by centrifugation at 5,000 ×*g* for 15 min.
9. Carefully collect the supernatant, filter (0.22 µm) and transfer to sterile tubes.
10. Store at 4 °C until further use.

S1.2.2. CsCl purification

1. Prepare the different CsCl solutions by dissolving the salt in SM buffer. The most commonly used solutions are listed in Table S1.

Table S1. CsCl solutions currently used for phage purification

d (g/mL)	*CsCl* (g/mL)	*n (refractive index)*
1.20	0.275	1.3527
1.25	0.342	1.3575
1.30	0.410	1.3622
1.40	0.546	1.3717
1.45	0.614	1.3765
1.50	0.683	1.3813
1.60	0.820	1.3908
1.70	0.959	1.4003

2. Add 0.5 g of solid CsCl per mL of phage sample and agitate until the salt is dissolved. Caution! Add small amounts of CsCl at a time and dissolve gradually, to avoid osmotic shock.
3. Prepare step gradients: Add the less dense CsCl solution to the ultracentrifuge tube. Then layer the other CsCl solutions by order of density (from less to most dense) using a long canular needle, placing it at the bottom of the tube (preferably pressed to the wall of the tube) so that the solution is added below the previous solution. Be careful inserting and removing the needle to avoid breaking the gradient. The preparation of the CsCl gradient should be performed at the same place where the ultracentrifuge is located, to avoid mixing of the CsCl gradient during movimentation.
4. Once the gradient is ready, carefully add the phage suspension to the top of the gradient.
5. Adjust weights to equilibrate the tubes for the ultracentrifuge.
6. Centrifuge at 87,000 ×*g* for 2–3 h at 4˚C.
7. Collect the phage band by puncturing the side of the tube with a hypodermic needle (Fig. P1.S1).

8. Remove the CsCl from the phage suspensions by dialysis at 4˚C, two or three times for 30 min against ca. 500 volumes of SM buffer or overnight against 2,000 volumes of SM buffer.
9. Store the dialyzed phage suspension at 4˚C with a few drops of chloroform (if the phage tolerates chloroform).
10. If required, to separate phages from contaminant RNA and DNA, a second CsCl centrifugation can be performed. For this, place the phage suspension from 9 in an ultracentrifuge tube and fill it with CsCl solution (density equal to 1.5 g/mL in SM buffer.
11. Centrifuge at 150,000 ×*g* for 24h at 4˚C.
12. Collect the band as described in 7, dialyze as in 8 to remove CsCl and store the sample at 4 °C.

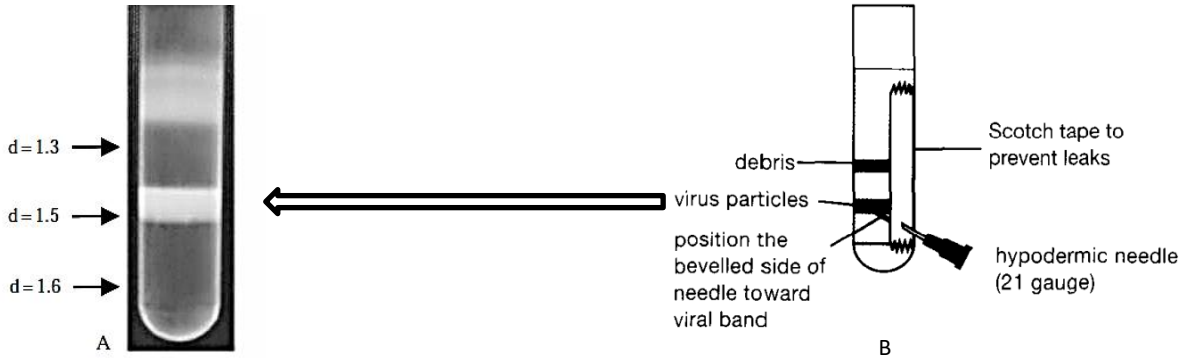


Fig. P1.S1. Purification of bacteriophages by CsCl centrifugation. (**A**) CsCl step gradient: phage (3 mL) layered over a CsCl step gradient: *d*= 1.6:2 mL, *d*= 1.5:3 mL, *d*=1.3:3 mL. After centrifugation in a rotor SW41, the phages form a bluish white and opalescent band located at the interface between the 1.4 and 1.5 or between the 1.5 and 1.6 density layers. (**B**) Collection of phages by side puncture (a tape attached outside the tube, level with the phage band, prevents leakage around the needle) (adapted from Sambrook and Russell[1]).

S1.3. NOTES

1. For example, thinwall Pollyallomer tubes (No. 331372 from Beckman-Coulter).

S1.4. REFERENCES

1. Sambrook J. and Russell D.W. (2001). Molecular cloning – a laboratory manual. Third ed: Cold Spring Harbor Laboratory.

PRACTICAL SESSION P2

Bacteriophage characterization

Carla Carvalho*, Diana Priscila Pires, Ana Oliveira, Catarina Milho, Maria Daniela Silva, Ergün Aktürk, Henrique Ribeiro, Rodrigo Monteiro
*carlacarvalho@deb.uminho.pt

1. INTRODUCTION

To assess the infectious potential of phages for different applications, several biological characterization assays can be performed. For example, the selection of phages to compose a cocktail to target a certain bacterial species relies on their lytic spectra that can be assessed spotting the phage on lawns of different bacterial strains. Usually serial dilutions of the phage stock are needed to observe individual phage plaques on the bacterial lawn. The main parameter influencing this assay is the efficiency of plating (EOP) of the phage, which may differ by several orders of magnitude on different host strains. EOA is the relative number of plaques that a phage stock is capable of producing on a certain host[1]. Phage growth parameters, such as the latent period and burst size are obtained through the one step growth curve[2,3,4], and depend on the phage host, growth conditions and phage type. Usually phages of the *Myoviridae* and *Siphoviridae* families give rise to lower burst sizes than *Podoviridae*. These growth parameters are important indicatives of how the phage interacts with the host and can be used in modelling to predict phage-host interactions[5].

Prior to performing genome sequencing of phages, researchers should have some information of their respective genome sizes. Furthermore, differences between phages can be confirmed by comparison of individual restriction fragment length polymorphism (RFLP) patterns[2,4].

2. MATERIALS

Prepare all solutions using distilled water. The media used in the procedures described herein is Tryptic Soy Broth (TSB) (see **note 1**) but alternative media can be used, depending on the bacterial species. Sterilize (autoclave at 121 °C for 15 min) all the solutions and material and store at room temperature (unless indicated otherwise).

2.1. Bacteriophage biological characterization

- TSB broth: Add 24 g of TSB to 800 mL of distilled water (see **note 1**)
- Bacterial culture in TSB
- Phage sample
- TSA plates (1.2 % (w/v) agar): Add 24 g of TSB and 9.6 g of agar to 800 mL of distilled water. After autoclaving pour on petri dishes
- Molten Top Agar (MTA_TSB) (0.6 % (w/v) agar): Add 15 g of TSB and 3 g of agar to 500 mL of distilled water. After autoclaving store accordingly (see **notes 2 and 3**)

- SM (saline magnesium) buffer: Prepare 1 M Tris-HCl buffer (pH 7.5) in a 100 mL bottle. Weigh 6.06 g of Tris-Base, add 50 mL of water and adjust the pH to 7.5 with HCl. Then, to a 1 L bottle, add 5.8 g of NaCl, 2 g of $MgSO_4.7H_2O$ and 50 mL of the prepared 1 M Tris-HCl (pH 7.5), and make up to 1 L with water (see **note 4**)
- 1.5 mL microcentrifuge tubes
- 15 mL centrifuge tubes
- 100 mL Erlenmeyer flasks

2.2. Bacteriophage DNA extraction, quantification and quality assessment

2.2.1. DNA extraction

- Phage samples (see **note 5**)
- Phage DNA extraction kit (see 3.2.1)
- Ultrapure water
- 1.5 mL microcentrifuge tubes

2.2.2. DNA quantification and quality assessment

- Phage DNA samples
- Ultrapure water
- Nanodrop (or a similar equipment)
- Agarose Gel electrophoresis system
- Agarose
- 1× TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0): Make 1 L of a 50× TAE stock solution. Weight 242 g of Tris-base, add 57.1 mL of acetic acid and 100 mL of 0.5 M sodium EDTA. Add distilled water to 1 L. To make 1× TAE dilute 20 mL of 50× TAE into 980 mL of distilled water
- DNA Marker (for example DNA-HindIII Digest)
- DNA Loading Dye (6×)
- Midori Green Nucleic Acid Stain (or other)
- Gel doc 2000 (Bio-Rad) (or other) for agarose gel analysis

2.3. RFLP

- Phage DNA samples
- Restriction enzymes
- 1.5 mL microcentrifuge tubes
- Agarose gel electrophoresis system
- Agarose
- 1× TAE
- DNA Marker (e.g. DNA-HindIII Digest)
- DNA Loading Dye (6×)
- Midori Green Nucleic Acid Stain (or other)
- Gel Doc 2000 (Bio-Rad) (or other) for agarose gel analysis

3. METHODS

3.1. Bacteriophage biological characterization

3.1.1. Lytic spectra

1. Grow bacterial strains in 100 mL Erlenmeyer flasks, containing 25 mL of TSB, until cells reach exponential phase (OD_{600}=0.5).
2. Pour 100 µL of bacterial suspensions into TSA plates, add 3-5 mL of MTA_TSB (47˚C) to form a uniform lawn, and let it dry for 2-5 min.
3. Spot, individually, 10 µL of each undiluted phage sample on the lawns and incubate overnight at optimal temperature (depending on the bacterial species).
4. Determine the lytic spectra of each phage by visual inspection of lysis clearing zones, i.e., the ability of phage to infect the host (Fig. P2.1A).

3.1.2. Efficiency of plating assay

1. Grow bacterial strains in 100 mL Erlenmeyer flasks, containing 25 mL of TSB, until cells reach exponential phase (OD_{600}=0.5).
2. Pour 100 µL of bacterial suspensions into TSA plates, add 3-5 mL of MTA_TSB (47 °C) to form a uniform lawn, and let it dry for 2-5 min.
3. Prepare ten-fold serial dilutions in SM buffer of the phage samples (add 100 µL of phage solution and 900 µL of SM buffer to 1.5 mL microcentrifuge tubes).
4. Spot, individually, 5 µL of each phage dilution, into the lawn and incubate at optimal temperature, overnight.
5. Determine the efficiency of plating (EOP), i.e. the relative phage titer on a bacterial strain compared to the maximum titer observed (Fig. P2.1B).
6. Score high, moderate and low efficiency of plating when phage titer for each strain represents 100-10 %, 1-0.1 % and 0.01-0.001 %, respectively.

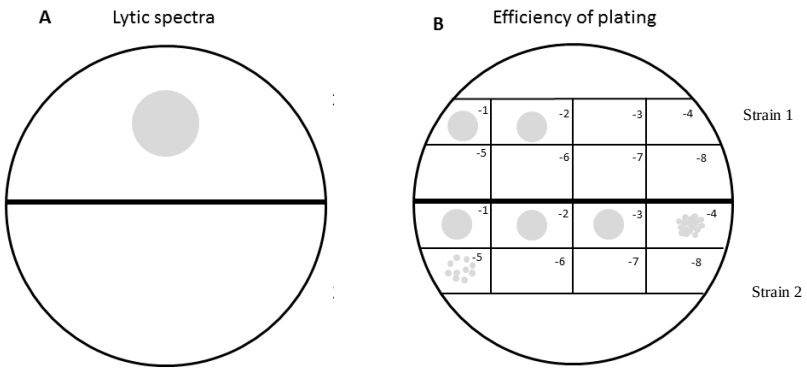


Fig. P2.1. Bacteriophage characterization. (**A**) Lytic spectra; and (**B**) Efficiency of plating.

3.1.3. One-step growth curve (OSGC) parameters

1. Grow bacteria in 100 mL Erlenmeyer flasks, containing 25 mL of TSB, until cells reach exponential phase (OD_{600} = 0.5).

2. Centrifuge 10 mL of suspension at 7,000 ×*g*, 5 min, 4 °C, and discard the supernatant.
3. Resuspend the pellet in 5 mL fresh media (OD_{600} of approx. 1.0; ≈10^8 CFU/mL).
4. Add 5 mL of phage with a titer of 8×10^5 PFU/mL to have the desired MOI (≤0.001) (see **note 6**).
5. Incubate with agitation for 5 min to allow the phages to adsorb to host cells.
6. Centrifuge the 10 mL culture at 7,000 ×*g*, 5 min, 4 °C, and discard the supernatant.
7. Resuspend the pellet in 10 mL of fresh TSB medium.
8. Incubate at proper temperature and under agitation (150-200 rpm).
9. Take a sample (100 µL) and immediately perform 10-fold serial dilutions and plate the sample. This sample will represent time zero of the experiment.
10. Take samples at 10 min intervals until 60 min and immediately dilute and plate the samples.
11. Plot the results to determine the phage latent period and burst size. The OSGC is better represented by the least squares, fitting the data to a typical sigmoidal curve (Fig. P2.2).

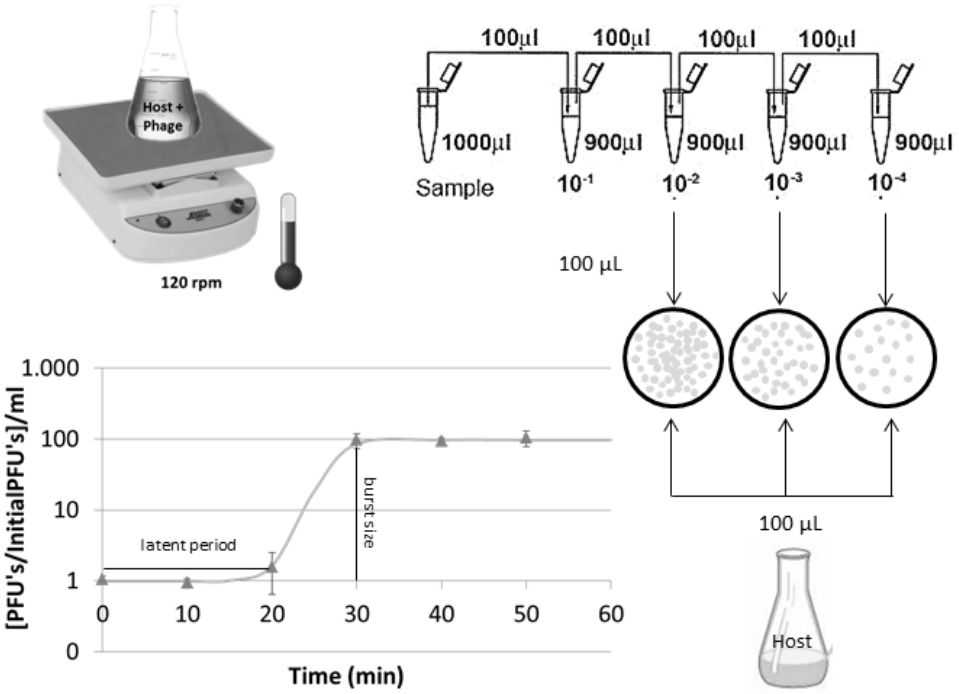


Fig. P2.2. One-step growth curve. Above, the 10-fold serial dilution and plating procedure to determine PFU/mL at different time points. Below, the graphical representation for determining the phage latent period and burst size.

3.2. DNA extraction, quantification and quality assessment

3.2.1. DNA extraction

There are several protocols for carrying out phage DNA extraction. Based on our experience we suggest two different approaches – a phenol:chloroform classic DNA extraction (see P2 Supplementary Protocol) or the use of commercial kits (Table P2.1). DNA extraction is performed according to the manufacturer instructions.

Table P2.1. Examples of commercial kits available for phage DNA extraction

Name	Company
GRS Viral DNA/RNA purification kit	Grisp-research solutions
Lambda DNA Purification Kit	Agilent Technologies
Wizard® DNA Clean-Up System	Promega
Phage DNA Isolation Kit	Norgen
Phage DNA Isolation Kit	Bio-world
ZR Viral DNA Kit™	Zymo Research

3.2.2. DNA quantification and quality assessment

Nanodrop

1. Open the Nanodrop software selecting the nucleic acids mode.
2. Place 1.5 µL of clean water onto the lower optic surface, selecting "initialize" in the software. Clean both optical surfaces with a tissue.
3. Perform a blank measurement with 1.5 µL of DNA elution buffer, selecting "blank" on software. Clean both optical surfaces with a tissue.
4. Measure the phage nucleic acid samples by loading 1.5 µL of each sample and selecting "measure". Clean both optical surfaces with a tissue after the measurement of each sample.
5. After measurement, Nanodrop software will provide sample concentration, as well as 260/280 and 260/230 ratios (see **note 7**).

Agarose Gel Electrophoresis

1. Setup the casting trays.
2. Weigh the appropriate amount of agarose (1 % (w/v)) and resuspend in TAE buffer.
3. Dissolve the agarose by heating in the microwave.
4. Cool the agarose solution and add the dye.
5. Pour the gel and let dry.
6. Add the appropriate volume of 6x loading dye to the samples and load them in the wells, as well as the DNA marker (5 µL).
7. Run the gel at 100 Volts (about 1 h) in 1× TAE (see **note 8**).
8. Use the Gel Doc 2000 (Bio-Rad) (or other) for agarose gel analysis.

3.2.3. RFLP

1. Digest around 3 µg of genomic DNA with 10 Units of each of the selected restriction enzymes in the appropriate buffer (see **note 9**).
2. Incubate the samples at the suitable temperature (check the temperature recommended by the manufacturer) for a minimum of 2 h.
3. Prepare a 0.6-0.8 % (w/v) agarose gel in 1× TAE, and add the dye.
4. Add the appropriate volume of 6x loading dye to the samples and load them in the wells; add the DNA marker.
5. Run the gel 2 h at 70 Volts (about 2 h) in 1x TAE (see **note 8**).
6. Use Gel Doc 2000 (Bio-Rad) (or other) for agarose gel analysis.

4. NOTES

1. TSB medium is a rich medium that is commonly used to culture members of the Enterobacteriaceae as well as for coliphage plaque assays. TSB is commercially available otherwise prepare as follows: to 1 L of distilled water add 17.0 g of Enzymatic Digest of Casein (Tryptone); 3.0 g of Enzymatic Digest of Soybean Meal (Soytone); 5.0 g of NaCl; 2.5 g of dipotassium phosphate (K_2HPO_4); and 2.5 g of Dextrose.
2. MTA_TSB is generally prepared with 0.6 % (w/v) of agar; however agar percentages between 0.4 % and 0.7 % can be used. Alternatively, MTA can be prepared with agarose instead of agar.
3. MTA_TSB can be stored at 47 °C if used within 1 day or at 4-21 °C. Solid MTA can be melted using a water bath or a microwave oven.
4. Optional: 2 % (w/v) of gelatin can be added to SM buffer. Gelatin is known to preserve phages and thus can be used in the later steps of phage purification.
5. It is recommended to work with high concentrations ($\geq 10^{10}$ PFU/mL) of phages purified by polyethylene glycol (PEG) or ideally cesium chloride to ensure extraction of pure DNA.
6. Multiplicity of infection (MOI) is defined by the ratio of the number of phage particles to the number of bacterial cells available in a sample.
7. Nucleic acids have maximum absorbance at 260 nm. A 260/280 ratio of ~1.8 is generally accepted as "pure" for DNA. 260/280 nm poor ratios may be a consequence of protein contamination. The 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values (2.0-2.2). 260/230 nonconforming results might be a consequence of contamination with organic compounds.
8. Reduced voltage increases gel resolution.
9. Select restriction enzymes that use the same buffer.

5. REFERENCES

1. Kutter E. (2009). Phage host range and efficiency of plating. *Methods Mol Biol*. **501**:141-9.
2. Sambrook J. and Russell D.W. (2001). Molecular cloning - a laboratory manual. Third ed: Cold Spring Harbor Laboratory.
3. Santos S.B., Carvalho C.M., Sillankorva S., Nicolau A., Ferreira E.C., Azeredo J. (2009). The use of antibiotics to improve phage detection and enumeration by the double-layer agar technique. *BMC Microbiol* **9:** 148.
4. Clokie M.R.J. and Kropinski A.M. (2009). Bacteriophages. Humana Press.
5. Santos S., Carvalho C.M., Azeredo J. (2014). Population Dynamics of a Salmonella Lytic Phage and Its Host: Implications of the Host Bacterial Growth Rate in Modelling. *PLoS ONE* **9**(7): e102507

P2 SUPPLEMENTARY PROTOCOL

S1. Phenol/Chloroform DNA extraction

DNA is best isolated from phage lysates by digesting the viral coat proteins with a protease such as proteinase K, followed by extraction with phenol:chloroform[1,2].

S1.1. MATERIALS

- Phage sample (see **note 1**)
- 1M Tris-HCl, pH 7.5: Dissolve 6.05 g of Tris base in 30 mL of distilled water. Adjust pH to 7.5 with HCl and fill up to 50 mL with distilled water
- 0.5 M EDTA, pH 8.0: Add 18.6 g EDTA to 80 mL of distilled water and stir the solution vigorously using a magnetic stirrer. Adjust pH to 8.0 using NaOH (the EDTA will slowly go into solution as the pH nears 8.0). Add distilled water to the final volume of 100 mL
- L1 Buffer: 300 mM NaCl; 100 mM Tris-HCl, pH 7.5; 10 mM EDTA; 0.2 mg/mL BSA; 20 mg/mL RNase A; 6 mg/mL DNase I: Add 0.175 g of NaCl, 1 mL of 1M Tris-HCl pH 7.5, 200 µL of 0.5 M EDTA, 2 mg of BSA, 200 mg of RNase A and 60 mg of DNase I to a 15 mL centrifuge tube and fill up to 10 mL with distilled water. Filter (0.22 µm) and store at 4 °C
- Proteinase K 10 mg/mL
- 10 % (w/v) SDS
- Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v)
- Chloroform
- Isopropanol
- 70 % (v/v) ethanol
- 3 M sodium acetate, pH 4.6: Add 40.82 g of $NaOAc.3H_20$ to 80 mL of distilled water. Adjust pH to 4.6 with glacial acetic acid. Add distilled water to the final volume of 100 mL
- Ultrapure water
- 1.5 mL microcentrifuge tubes
- Water bath or heating plate with controlled temperature

S1.2. METHODS

1. Add 0.016 % (v/v) L1 Buffer to the purified phages and incubate for 2 h at 37 °C.
2. Incubate the sample for 15 min at 70 °C to inactivate the enzymes.
3. Add 1 % (w/v) SDS, 20 mM EDTA and 50 µg/ml Proteinase K and incubate 2 h to overnight at 65 °C.
4. Cool down to room temperature.
5. Add 1 vol of Phenol:Chloroform:Isoamyl alcohol (25:24:1) and centrifuge at 3,000 x*g*, 15 min, room temperature.
6. Carefully recover the upper phase (see **note 2**).
7. Repeat steps 5 and 6.
8. Add 1 vol of chloroform and centrifuge at 1,600 x*g*, 15 min, room temperature.

9. Carefully recover the upper phase (see **note 2**).
10. Add 0.1 vol of 3 M sodium acetate (pH 4.6) and 0.8 vol of isopropanol.
11. Incubate on ice for 30 min.
12. Centrifuge at 12,600 x*g*, 10 min, 4 °C.
13. Wash the pellet with 70 % (v/v) ethanol.
14. Air dry the pellet for a minimum of 30 min.
15. Resuspend the pellet in 50 µL ultrapure water.
16. Store at -20 °C.

S1.3. NOTES

1. It is recommended to work with high concentrations (≥10^{10} PFU/mL) of phages purified by PEG or ideally cesium chloride to ensure extraction of pure DNA.
2. Carefully avoid pipetting any flocculent material at the interface. This material contains proteins and other debris, which should be avoided for subsequent assays. Tip: set your pipette to only recover 80-90 % of the aqueous phase in the initial extraction.

S1.4. REFERENCES

1. Sambrook J. and Russell D.W. (2001). Molecular cloning - a laboratory manual. Third ed: Cold Spring Harbor Laboratory.
2. Clokie M.R.J. and Kropinski A.M. (2009). Bacteriophages. Humana Press.

PRACTICAL SESSION P3

Bacteriophage genome annotation

Sílvio Santos*, Hugo Oliveira, Luís Melo, Sanna Sillankorva
*silviosantos@deb.uminho.pt

1. INTRODUCTION

We live in a sequencing revolution era where the landmark of over 1,200 completely sequenced bacteriophage genomes has already been accomplished (http://www.ebi.ac.uk/genomes/phage.html). The fast-declining per-base sequencing costs has created an uncontrolled influx of DNA sequences encouraging laboratory scientists to engage large datasets in comparative sequence analyses for making evolutionary functional and translational inferences[1]. Despite a constant increase in the number of phage sequences in the public databases, few functional genomics studies of phages have been conducted. 'Functional genomics' in phages comprises a range of aspects: sequencing of phage genomes, annotation of phage gene functions, identification of prophages in bacterial sequences, elucidation of events in various stages of phage life cycle using genomics, transcriptomics and proteomics approaches, definition of the mechanisms of host takeover including specific bacterial-phage protein interactions, and identification of virulence and other adaptive features encoded by phages[2].

2. METHODS

Several user-friendly software are currently available and were designed and developed to fulfill the needs of biologists to analyze large datasets. Most of the software programs use sophisticated computational methods and Table P3.1 provides a brief catalogue of some analysis tools, many of which will be approached in this Bacteriophage Genome Annotation session.

Table P3.1. Examples of genomic analysis software, tools and services available

Name	Description	Website	License & cost
ARAGORN	Search for transfer RNA (tRNA) and show their positioning.	http://mbio-serv2.mbioekol.lu.se/ARAGORN/	Free
ARNold	Search for transcription terminators (e.g. rho-independent terminators) in nucleic acid sequences.	http://rna.igmors.u-psud.fr/toolbox/arnold/	Free
Bionumerics	Search, organize and analyze genome and protein information (e.g. assembly and annotation).	http://www.applied-maths.com/bionumerics	License of €7,000
BLAST	Search for regions of similarity between biological sequences. Compare nucleotide or protein	https://blast.ncbi.nlm.nih.gov/Blast.cgi	Free

Name	Description	Website	License & cost
	sequences to sequence databases and calculate the statistical significance.		
CLC Genomic Workbench	Search, organize and analyze genomic and protein information (e.g. assembly, alignment, annotation and prediction, etc.)	https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/	12-day trial. Academic and non-academic license.
Compute pI/Mw	Calculate the isoelectric point (pI) and molecular weight (MW) of proteins.	http://web.expasy.org/compute_pi/	Free
CoreGenes	Analyze genes or proteins in common from sets of up to five genomes to find conservation.	http://binf.gmu.edu:8080/CoreGenes3.0	Free
Geneious	Search, organize and analyze genomic and protein information (e.g. assembly, alignment, annotation and prediction, etc.).	http://www.geneious.com/	15-day trial. Academic and non-academic license.
HHMER	Search sequence databases for sequence homologs, and sequence alignments.	http://hmmer.org/	Free
HHpred	Detect homology, and predict protein function and structure by HMM-HMM comparison.	https://toolkit.tuebingen.mpg.de/#/tools/hhpred	Free
InterProScan	Predict protein domains. Scan a sequence for matches against the InterPro protein signature databases.	http://www.ebi.ac.uk/interpro/search/sequence-search	Free
MEME/MAST	Predict putative transcription promoters and discover conserved motifs.	http://meme-suite.org/doc/mast.html	Free
Mfold	Predict the folding structure of terminator sequences.	http://unafold.rna.albany.edu/?q=mfold	Free
myRAST	*In silico* automatic annotation of genomes.	http://blog.theseed.org/servers/presentations/t1/drast-overview.html	Free
OrthoVenn	Genome wide comparison and annotation of orthologous clusters across multiple species.	http://www.bioinfogenome.net/OrthoVenn/	Free
Pfam 27.0	Search for protein families.	http://pfam.xfam.org/	Free
PHIRE	Predict putative transcription promoters by performing an algorithmic string-based search on phage genome sequences, discovering sequence similarity corresponding to conserved regulatory elements.	http://www.agr.kuleuven.ac.be/logt/PHIRE.htm	Free
Phobius	Search for transmembrane domains in proteins.	http://phobius.sbc.su.se/	Free
TMHMM	Search for transmembrane domains in proteins.	http://www.cbs.dtu.dk/services/TMHMM/	Free

Name	Description	Website	License & cost
TransTermHP	Search for transcription terminators (e.g. rho-independent terminators) in nucleic acid sequences.	http://transterm.cbcb.umd.edu/	Free
tRNAscan-SE	Search for tRNA and show their positioning.	http://lowelab.ucsc.edu/tRNAscan-SE/	Free
SignalP	Predict the presence and location of signal peptide cleavage in amino acid sequences.	http://www.cbs.dtu.dk/services/SignalP/	Free

Although the software programs described in Table P3.1 are extremely useful, the generated data should always be checked manually.

3. REFERENCES

1. Kumar, S. and Dudley, J. (2017). Bioinformatics software for biologists in the genomics era. *Bioinformatics*, **23**(14): 1713-1717.

2. Klumpp, J. (2013). Bacteriophage functional genomics and its role in bacterial pathogen detection. *Brief Funct Genomics*, **12**(4): 354-365.

PRACTICAL SESSION P4

Characterization of bacteriophage endolysins

Hugo Oliveira*, Sílvio Santos, Luís Melo, Graça Pinto
*hugooliveira@deb.uminho.pt

1. INTRODUCTION

Endolysins are phage encoded enzymes produced at the end of the phage lytic cycle that cleave the peptidoglycan. When working with recombinant endolysins, the enzymes need to reach the peptidoglycan from without. While this structure is exposed in Gram-positive bacteria, in Gram-negative cells there is a protective outer membrane that blocks the entry of the endolysins, preventing peptidoglycan digestion and subsequent lysis of the cells. Therefore, protoplasts (cells without outer membrane) need to be used to assess enzyme activity. In terms of their molecular organization, endolysins can either be globular, with one catalytic domain, or modular having an extra cell wall binding domain. Herein, we describe the characterization of two endolysins (globular and modular) from phages infecting Gram-negative and Gram-positive hosts, respectively. The characterization involves: i) a spot-on-lawn method to detect the presence/absence of activity; ii) a kinetic assay to assess the optimal physiological conditions for enzymatic activity (e.g. ionic strength and pH); and iii) a cell wall binding assay, to detect the peptidoglycan binding domain through its fusion with a Green Fluorescent Protein (GFP). Several studies reporting these tests can be found in the literature[1,2,3,4].

2. MATERIAL

Prepare all solutions using distilled water. The media used in the procedures described herein is Tryptic Soy Broth (TSB) (see **note 1**) but alternative media can be used, depending on the bacterial species. Sterilize (autoclave at 121 ˚C, 1 bar, for 15 min) all the solutions and material and store at room temperature (unless indicated otherwise).

2.1. Detection of endolysin activity

- TSA agar plates (1.2 % (w/v) agar): Add 24 g of TSB and 9.6 g of agar to 800 mL of distilled water. After autoclaving pour on petri dishes
- Molten Top Agar (MTA_TSB) (0.6 % (w/v) agar): Add 15 g of TSB and 3 g of agar to 500 mL of distilled water. After autoclaving store accordingly (see **notes 2 and 3**)
- 20 mM Tris buffer, pH 8.0: Dissolve 1.21 g of Tris in 400 mL of distilled water. Adjust pH to 8.0 with HCl and bring the final volume to 500 mL
- 1.5 mL microcentrifuge tubes
- 100 mL Erlenmeyer flasks
- 2 µM endolysin stock

2.2. Optimization of endolysin activity

- TSB broth: Add 24 g of TSB to 800 mL of distilled water (see **note 1**)
- TSA plates
- MTA_TSB at 47 ˚C (see **notes 2 and 3**)
- 20 mM Tris buffer, pH 8
- Sodium citrate buffer, pH 4.0: Add 2.58 g of sodium citrate to 400 mL of distilled water. Adjust pH to 4.0 and bring the final volume to 500 mL
- Sodium citrate buffer, pH 6.0: Add 2.58 g of sodium citrate to 400 mL of distilled water. Adjust pH to 6.0 and bring the final volume to 500 mL
- Boric acid buffer, pH 10: Add 0.62 g of boric acid to 400 mL of distilled water. Adjust pH to 10 and bring the final volume to 500 mL
- 15 mL microcentrifuge tubes
- 100 mL Erlenmeyer flasks
- Endolysin stock

2.3. Detection of the endolysin peptidoglycan binding domain

- TSB broth (see **note 1**)
- 100 mL Erlenmeyer flasks
- Fluorescence microscope
- Microscope slides
- Coverslips
- 20 mM Tris buffer
- 1.5 mL microcentrifuge tubes
- Cell wall binding domain fused with GFP

3. METHODS

Carry out all procedures at room temperature, unless otherwise specified. Please note that on 3.1 we will use phage endolysins of Gram-positive and Gram-negative hosts, while in 3.2 and 3.3 we will use either the endolysin of the Gram-positive host or its cell wall binding domain fused with a GFP, against *Paenibacillus larvae*.

3.1. Detection of endolysin activity

1. Add 100 µL of a bacterial culture grown overnight, and 3-5 mL of MTA_TSB (47 ˚C) to a TSA plate and gently mix.
2. Let the plates dry and incubate at 37˚C until the lawn is formed (≈ 4 h).
3. For Gram-negative hosts, permeabilize the outer membrane (see P4 Supplementary Protocol S1).
4. Prepare ten-fold serial dilutions in Tris buffer from a 2 µM endolysin stock (add 20 µL of endolysin solution to 180 µL of 20 mM Tris buffer in 1.5 mL microcentrifuge tubes).
5. Add a drop of 10 µL of the dilution mixture onto the formed lawn.
6. Let the plates stand still until the drops have completely dried.

7. Incubate a few minutes at room temperature.
8. Visualize the presence/absence of lysis haloes formed within the drop.

3.2. Optimization of endolysin activity

1. Grow cells in 100 mL Erlenmeyer flasks containing 25 mL of TSB, until exponential phase (OD_{600} = 0.5).
2. For Gram-negative bacteria, prepare protoplasts accordingly (see P4 Supplementary Protocol S2) and proceed to step 5.
3. For Gram-positive bacteria, centrifuge individually 1 mL of suspensions (one for each buffer to be tested) at 7,000 ×*g*, 5 min, 4 ˚C, and discard the supernatant.
4. Resuspend the pellet in 20 mM of different buffer systems: 20 mM sodium citrate (pH 4 and 6); 20 mM Tris (pH 8) and 20 mM boric acid (pH 10) till an OD_{600} = 1.0.
5. Add 180 µL of each cell suspension into a microtiter plate and add 20 µL of the endolysin at 2 µM end concentration.
6. Check the OD_{600} over time.
7. The activity can be determined by comparing the kinetic maximum slopes or the final OD achieved (Fig. P4.1).

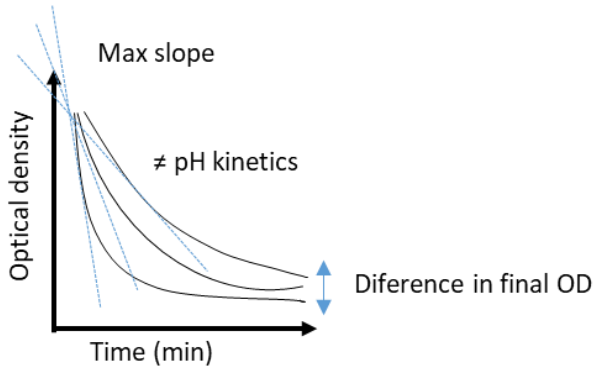


Fig. P4.1. Lytic spectra and kinetic curve graphic representations.

3.3. Detection of the endolysin peptidoglycan binding domain

1. Grow bacterial strains in 100 mL Erlenmeyer flasks, containing 25 mL of TSB, until exponential phase (OD_{600} = 0.5).
2. Pour 100 µL of bacteria into 1.5 mL microcentrifuge tubes and add 900 µL of 20 mM Tris buffer.
3. Centrifuge at 7,000 ×*g*, 2 min at 4˚C and discard the supernatant.
4. Resuspend in 100 µL of 20 mM Tris buffer.
5. In a different 1.5 mL microcentrifuge tube add 20 µL of the bacterial suspension and 20 µL of the cell wall binding domain fused with GFP at 2 µM end concentration (test experiment).
6. Incubate in the dark for 15 min.
7. Add 900 µL of 20 mM Tris buffer and centrifuge at 7,000 ×*g*, 2 min at 4 ˚C. Discard the supernatant.
8. Repeat the previous step.
9. Spin down solutions (7,000 ×*g*, 10 s).
10. Add 5 µL of solutions into the microscopic slides, let dry, and cover with a coverslip.
11. Observe under the fluorescence microscope.

4. NOTES

1. TSB medium is a rich medium that is commonly used to culture members of the Enterobacteriaceae as well as for coliphage plaque assays. TSB is commercially available otherwise prepare as follows: to 1 L of distilled water add 17.0 g of Enzymatic Digest of Casein (Tryptone); 3.0 g of Enzymatic Digest of Soybean Meal (Soytone); 5.0 g of NaCl; 2.5 g of dipotassium phosphate (K_2HPO_4); and 2.5 g of Dextrose.
2. MTA_TSB is generally prepared with 0.6 % (w/v) of agar; however agar percentages between 0.4 % and 0.7 % can be used. Alternatively, MTA can be prepared with agarose instead of agar.
3. MTA_TSB can be stored at 47 °C if used within 1 day or at 4-21 °C. Solid MTA can be melted using a water bath or a microwave oven.

5. REFERENCES

1. Oliveira H, Vilas Boas D, Mesnage S, Kluskens LD, Lavigne R, Sillankorva S, Secundo F, Azeredo J. (2016). Structural and enzymatic characterization of ABgp46, a novel phage lysin with broad anti-Gram-negative bacterial activity. *Front Microbiol*. **7**:208.
2. Oliveira H, Thiagarajan V, Walmagh M, Sillankorva S, Lavigne R, Neves-Petersen M, Kluskens LD, Azeredo J. (2014). A thermostable Salmonella phage endolysin, Lys68, with broad bactericidal properties against gram-negative pathogens in presence of weak acids. *PLoS One*. **9**: e108376
3. Oliveira A, Leite M, Kluskens L, Santos, S, Melo L, Azeredo J. (2015). The first *Paenibacillus larvae* bacteriophage endolysin (PlyPl23) with high potential to control American foulbrood. *PLoS One*, **10**(7): e0136331.
4. Oliveira H, Pinto G, Oliveira A, Oliveira C, Faustino M, Briers Y, Domingues L, Azeredo J. (2016). Characterization and genome sequencing of a *Citrobacter freundii* phage CfP1 harbouring a lysin active against multidrug-resistant isolates. *Appl Microbiol Biotechnol*. **24**:100.

P4 SUPPLEMENTARY PROTOCOLS

S1. Preparation of protoplasts on lawns to detect phage endolysin activity on Gram-negative bacteria

This is the first basic test after the recombinant production of a new endolysin. After growth of a bacterial lawn, the bacterium outer membrane must be permeabilized prior to dropping and visualizing the presence/absence of endolysin activity. This procedure can be applied to most bacteria without modification.

S1.1. MATERIALS

- TSB (see note 1 of P4)
- TSA plates
- MTA_TSB (see note 2 and 3 of P4)
- Chloroform
- 100 mL Erlenmeyer flasks
- 2 µM endolysin stock

S1.2. PROCEDURE

1. Make an overnight culture from a single colony on TSB.
2. Add 100 µL of bacterial culture grown overnight, and 3-5 mL of MTA_TSB (47 ˚C) to a TSA plate and gently mix.
3. Incubate at 37 ˚C to allow lawn formation.
4. Expose overlay plates containing the bacterial host to 30 min of chloroform vapor by inverting plates onto lids containing 3 mL of chloroform.
5. Spot the endolysin (20 µL at 2 µM) onto overlay surface of chloroform exposed plates and allow them to dry.
6. Incubate plate at room temperature. Lysis can be detected in a few minutes.

S2. Preparation of protoplasts on suspension to optimize phage endolysin activity on Gram-negative bacteria

This is the second test to evaluate endolysin activity on Gram-negative bacteria. Herein we show how to prepare protoplasts in suspensions (bacterial cell without outer membrane) to monitor the kinetic of the peptidoglycan degrading activity of endolysins. This procedure has been previously published and can be applied to most bacteria without modifications[1].

S2.1. MATERIALS

- TSB (see note 1 of P4)
- Petri dishes
- 80 mM phosphate buffer, pH 7.3: Add 1.22 g of KH_2PO_4 and 5.36 g of K_2HPO_4 to 1 L of distilled water
- Centrifuge bottles
- 50 mM Tris-HCl, pH 7.7: Add 6.06 g of Tris-base to 800 mL of distilled water. Adjust pH to 7.7 using HCl. Add distilled water to a final volume of 1 L
- Chloroform
- 15 mL centrifuge tubes

S2.2. PROCEDURE

1. Inoculate the bacterium in 600 mL warm TSB with 6 mL of overnight culture.
2. Incubate at 37 °C until OD_{600} = 0.6 (about 2.5 h).
3. Centrifuge cells at 4,600 x*g* for 30 min at 4 °C.
4. Prepare chloroform saturated Tris, mixing 300 mL of chloroform and 400 mL of 50 mM Tris-HCl, pH 7.7 and shake vigorously. Make it just after starting to centrifuge cells.
5. Resuspend pellet in 240 mL chloroform-saturated Tris pipetting from the upper layer.
6. Shake the suspension gently at room temperature for 45 min to disrupt the cell wall. Time starts directly after adding the chloroform-saturated buffer. Balance the different centrifuge bottles before starting to shake the tubes to prevent time loss when you start the centrifugation after 45 min.
7. Centrifuge 4,600 x*g* for 30 min at 4 °C, resuspend pellet in 90 mL of 80 mM Phosphate pH 7.3, and divide in different 15 mL centrifuge tubes (you may resuspend in different buffers to study the pH or the ionic strength effect on the endolysin activity).
8. Repeat centrifugation and resuspend pellet in Phosphate buffer pH 7.3 until $OD_{600} \sim 1.5$.
9. Store cells at -20 °C prior to use.

S2.3. REFERENCES

1. Briers Y, Lavigne R, Volckaert G, Hertveldt K. 2007. A standardized approach for accurate quantification of murein hydrolase activity in high-throughput assays. *J Biochem Biophys Methods* **70**: 531–533.

PRACTICAL SESSION P5

Phage display technology

Ivone Martins*, Ana Rita Costa, Débora Ferreira
*ivone.martins@ceb.uminho.pt

1. INTRODUCTION

Phage display, introduced in 1985 by George P. Smith using filamentous bacteriophage as an expression vector[1], is a powerful, high-throughput technology used to identify interacting molecules and ligands for a given target. It has been successfully employed to identify peptide ligands for a wide variety of targets, ranging from relatively small molecules (enzymes, cell receptors) to inorganic, organic and biological (tissues) materials[2-5]. The concept is simple: a library of phage particles expressing a wide diversity of peptides is used to select those that bind the desired target. Peptides, fused to capsid proteins on the surface of phages and coupled with *in vitro* selection, enable rapid identification of peptide sequences. It is an elegant approach whereby the products of a gene, harbored within the genome of the phage are found on the surface of the virus particle (virion). The genes encoding the protein product are found inside the virion while the protein is displayed on its surface. This combination allows the selection of the protein on the phage surface, while the sequence of the gene found inside the particle encoding that protein can be analyzed.

In the last two decades, phage display technology has advanced tremendously and has become a powerful tool in varied fields of research, including biotechnology (separation processes, enzyme assays, selection of new antibodies), materials science (surface functionalization, self-assembly, nanomaterials), cell biology (protein-protein interactions that underlie cellular processes, antibodies for cell- or tissue-specific markers), pharmacology (drug discovery and design, vaccine development, targeted therapy) and diagnosis (molecular recognition, analytical reagents for biosensing, probes for imaging)[6-11].

The growing interest and success of phage display is largely due to the incredible versatility and practical use of the libraries. A phage display library is a collection of independent clones, each carrying a different foreign DNA insert in the phage genome. The foreign gene sequence encoding an antibody, protein or peptide, is spliced between genes encoding a phage signal peptide and a portion of the coat protein, which ensures that the foreign protein is produced as a fusion with the coat protein. Molecules displayed on phage libraries are not limited to peptides and antibodies. cDNA, mRNA phage display libraries and libraries of random protein fragments have been also created[12,13,14] expanding the practical applications of the technology.

A different approach of the conventional screening, selection and sorting of cell-surface-binding peptides from phage libraries, called Biopanning and Rapid Analysis of Selective Interactive Ligands (BRASIL), was introduced by Giordano and co-workers[15]. The technique allows separation of phage-cell complexes from the remaining unbound phage using differential centrifugation. Here a cell suspension incubated with the phages in an aqueous upper phase is centrifuged through a non-miscible organic lower phase (Fig. P5.1). Cells are driven from a hydrophilic environment into a non-miscible organic phase; because the organic phase is hydrophobic, it excludes water-soluble materials surrounding cell surfaces.

Bound phages are recovered from the cell pellet whereas the unbound phages remain soluble in the upper aqueous phase. This single-step organic phase separation is faster, more sensitive and more specific than conventional methods that rely on washing steps or limiting dilution. This practical class will be based in adapted procedures from Arap and Kiessling groups[9,15].

2. MATERIALS

Prepare all solutions using distilled water. The media used in the procedures herein is Tryptic Soy Broth (TSB) (see **note 1**). Sterilize (autoclave at 121 °C, 1 bar, for 15 min) all solutions and material and store at room temperature (unless indicated otherwise).

2.1. Cell detachment

- Cell lines:
  - Normal cell line (control cells for pre-clearing): MCF-10-2A (ATCC® CRL-10781™)
  - Breast cancer cell line (target cells for screening): MDA-MB-231 (ATCC® HTB-26™)
- Trypsin-EDTA solution: 0.05 % (w/v) trypsin: 0.02 % (w/v) EDTA
- 10× Phosphate Buffered Saline (PBS), pH 7.4: Weight 20 g of NaCl, 0.5 g of KCl, 3.6 g of Na_2HPO_4, 0.6 g of KH_2PO_4 and place in a 250 mL bottle. Add distilled water up to 200 mL and adjust the pH to 7.4 with HCl. Adjust the volume to 250 mL with distilled water
- 1× PBS, pH 7.4. Add 100 mL of the 10× PBS solution to 900 mL of sterile distilled water
- 25 cm^2 tissue culture flasks (T-25)
- Sterile plastic Pasteur pipettes
- Serological pipettes of 5 mL and 10 mL
- 1.5 mL microcentrifuge tubes
- 15 mL centrifuge tubes
- Neubauer chamber
- Coverslips
- Micropipettes and tips
- Microscope
- Centrifuge

2.2. BRASIL - Biopanning and Rapid Analysis of Selective Interactive Ligands

- Dulbecco's Modified Eagle Medium (DMEM) + 1 % (w/v) Bovine Serum Albumin (BSA): To a 50 mL bottle add 50 mL of DMEM and 0.5 g BSA. Dissolve by heating in a 37 °C water bath. Filter sterilize (0.22 µm) and store at 4 °C
- Dulbecco's Modified Eagle Medium (DMEM) + 3 % (w/v) Bovine Serum Albumin (BSA): To a 50 mL bottle add 50 mL of DMEM and 1.5 g of BSA, respectively. Dissolve by heating in a 37 °C water bath. Filter sterilize (0.22 µm) and store at 4 °C
- Organic phase 9:1 (v/v) dibutyl phthalate:cyclohexane: Mix 9 mL dibutyl phthalate and 1 mL cyclohexane.
- 1 M Tris-HCl, pH 9.1**:** For 250 mL weight 30 g of Tris base and add distilled water up to 200 mL. Adjust pH to 9.1 with HCl. Adjust the volume to 250 mL with distilled water
- 50 mL centrifuge tubes

- 15 mL centrifuge tubes
- 0.22 µm PES filters
- PhD-12 library (New England Biolabs)

2.3. Phage Titer Assay

- TSB: Add 12 g of TSB to 400 mL of distilled water (see **note 1**)
- 0.7 % (w/v) Molten Top Agar (MTA_TSB): Weight 12 g of TSB and 2.8 g of agar and add distilled water up to 400 mL. After autoclaving store accordingly (see **notes 2 and 3**).
- TSB+X-Gal+IPTG plates: Weight 12 g of TSB and 8 g of agar and add distilled water up to 400 mL. After autoclaving let the temperature drop to 50 °C and add 800 µL of 20 mg/mL X-Gal and 80 µL of 1 M IPTG. Pour on petri dishes. Store at 4 °C
- *Escherichia coli* K12 strain ER2738
- 15 mL centrifuge tubes
- 1.5 mL microcentrifuge tubes

2.4. ssDNA Isolation

- TSB (see **note 1**)
- *E. coli* ER2738
- 20 % (w/v) PEG/2.5 M NaCl: For 50 mL weight 7.3 g NaCl, 10 g polyethylene glycol (PEG) MW=8000 and add distilled water up to 50 mL. Dissolve and filter sterilize (0.22 µm). Store at room temperature
- Iodide Buffer: Add 8 mL of 5 M sodium iodide (NaI), 100 µl of 1 M Tris-HCl pH 8, 20 µl of 0.5 M EDTA pH 8 and distilled water up to 10 mL. Filter sterilize (0.22 µm) and store at 4 °C protected from light
- 100 % ethanol (cold)
- 70 % (v/v) ethanol in water (cold)
- 10× TE (Tris-EDTA buffer): Add 500 µL of 1 M Tris-HCl pH 8, 100 µL of 0.5 M EDTA pH 8 and distilled water up to 50 mL
- 1× TE, pH 8: Dilute 10× TE in distilled water to obtain a 1x solution and store at room temperature
- 15 mL centrifuge tubes
- 1.5 mL microcentrifuge tubes
- Sterile toothpicks
- 0.22 µm PES filters

3. METHODS

3.1. Cell detachment

1. Grow the control and target cell lines in T-25 flasks in 5 mL of the appropriate medium in a CO_2 incubator at 37 °C and 5 % of humidity.
2. When the cells reach approximately 80 % of confluence remove the medium (use a Pasteur pipette) and wash the cells with 1 mL PBS 1× pH 7.4.
3. Detach the cells using 500 µL of trypsin-EDTA solution and incubate in the CO_2 incubator for 5 min or until all cells are detached.

4. Add 500 µL of DMEM + 1 % BSA to remove all cells from the flask surface and transfer the suspension to a 15 mL centrifuge tube.
5. Put 10 µL in a Neubauer chamber to determine the cell number according to equation P5.1:

   Equation P5.1
   N (cell number) = number of cells counted / number of squares x 10,000 x volume

   The cell concentration must be adjusted to around $1x10^6$ cells/mL.
6. Centrifuge the cell suspension for 5 min at 1,400 rpm at room temperature.
7. Remove the supernatant and add the same volume of DMEM + 1 % (w/v) BSA.
8. Repeat steps 6 and 7 twice.
9. Remove all the supernatant and resuspend the pellet with 200 µl of pre-chilled DMEM + 3 % (w/v) BSA and transfer to a 1.5 mL microcentrifuge tube. Keep cells on ice.

3.2. BRASIL - Biopanning and rapid analysis of selective interactive ligands

3.2.1. Pre-clearing step (control cells)

1. Add 10 µL of the 10^{13} PFU/mL PhD-12 library and incubate on ice for 2 h, Fig. P5.1a.
2. Prepare the "BRASIL tube" [excise the bottom of a 2 mL polystyrene cryopreservation tube using a red-hot spatula, and place the resulting tube ("inner tube") inside a 15 mL centrifuge tube], containing the organic phase 9:1 (v/v) cyclohexane:dibutyl phthalate submerging the inner tube.
3. Add 500 µL of pre-chilled DMEM + 1 % (w/v) BSA on top of the organic layer in the inner tube such that it forms an aqueous droplet.
4. Carefully add the phage and cell suspension mixture inside the aqueous drop in the inner BRASIL-tube.
5. Centrifuge for 10 min at 10,000 *g* and 4 °C (Fig. P5.1b) and recover the droplet (phages that didn't bound to the cells) to a new 1.5 mL tube and store at 4 °C for titer analysis, Fig. P5.1c.

3.2.2. Screening step (target cells)

1. Add the unbound phage pool obtained from the supernatant (droplet) of the pre-clearing step to 200 µl of the target cells at $1x10^6$ cells/mL, and incubate on ice for 4 h, Fig. P5.1c.
2. Prepare the "BRASIL tube" as described for the pre-clearing step.
3. Add 500 µL of pre-chilled DMEM + 1 % (w/v) BSA on top of the organic layer in the inner tube so that it forms an aqueous droplet.
4. Carefully add the phage and cell suspension mixture inside the aqueous drop in the inner BRASIL-tube.
5. Centrifuge for 10 min at 10,000 x*g* and 4 °C. Remove the droplet and the organic phase and resuspend the pellet with 50 µL of 1 M Tris-HCl buffer pH 9.1. Put in a new 1.5 mL microcentrifuge tube and store at 4 °C for phage titer, Fig. P5.1e.

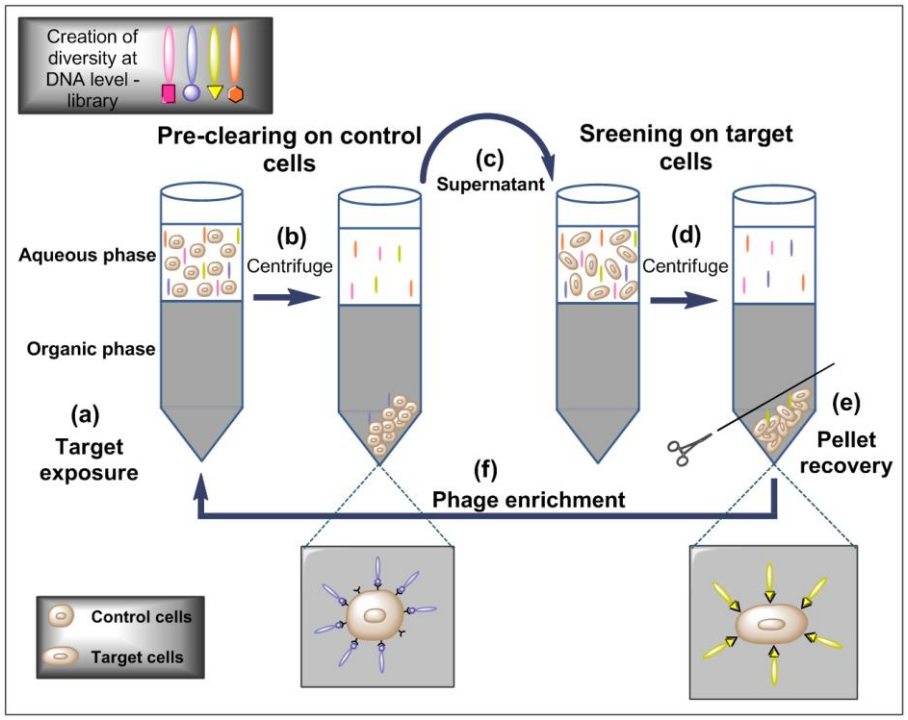
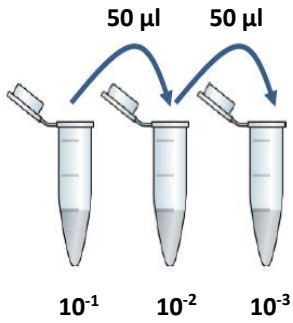


Fig. P5.1. Schematic representation of the BRASIL methodology. (**A**) Healthy cells are incubated with the phage library; (**B**) Cells are separated by centrifugation through the organic phase; (**C**) The supernatant, containing the unbound phage, is transferred to target cells (cancer cells); (**D**) After incubation, cells and bound phage are separated by centrifugation through the organic phase; (**E**) The cell-bound phage in the pellet is rescued by infection in bacteria; and (**J**) Amplified and used for another round of biopanning. After 3-4 rounds, individual clones are isolated and sequenced. Adapted from [15].

3.3. Phage titer assay

1. Inoculate 5 mL of TSB with ER2738 from a plate and incubate at 37 °C with shaking for 5 h until mid-log phase, $OD_{600} \approx 0.5$.
2. Prepare successive 10^{-1}, 10^{-2} and 10^{-3}-fold serial dilutions of the eluted phage in TSB (add 50 µL of the eluted phage and 450 µL of TSB in 1.5 mL microcentrifuge tubes) as follows:



3. In new 1.5 mL microcentrifuge tubes add 10 µL of the dilutions to 200 µL of *E. coli* ER2738, mix and incubate 1-5 min.
4. In a 15 mL centrifuge tube add 3 mL of 0.7 % (w/v) MTA_TSB and all the previous suspensions (10 µL of the dilutions + 200 µL of *E. coli* ER2738).

5. Vortex briefly and **IMMEDIATELY** pour culture onto a 37 °C pre-warmed TSA plate containing X-Gal and IPTG. Let it dry and incubate overnight at 37 °C.
6. Count the phage plaques in the dilution which resulted in 20-200 plaques and determine the titer of triplicate preparations according to equation P5.2:

Equation P5.2

$$Bacteriophage\ titer\ (PFU\ per\ mL) = \frac{Nr.of\ bacteriophage\ plaques\ formed\ \times Dilution\ factor}{Volume\ of\ bacteriophage\ sample\ (mL)}$$

NOTE: An aliquot of the eluted phage will be used for titer analysis. If necessary (when the titer is low), the rest will be amplified in E. coli ER2738, and purified by precipitation with polyethylene glycol 8000. An aliquot of the amplified phage will be subsequently reapplied to newly trypsinized target cells for a total of 4-5 biopanning rounds, ***Fig. P5.1f****.*

3.4. ssDNA isolation

1. Dilute an overnight culture of ER2738 1:100 in TSB and dispense 1 mL into 15 mL centrifuge tubes, one for each clone to be characterized.
2. Use a sterile toothpick to stab a blue plaque from a tittering plate (see **note 4**) and transfer to a tube containing the diluted culture. Incubate the tubes at 37 °C with shaking for 4.5 h.
3. Transfer the cultures to 1.5 mL microcentrifuge tubes and centrifuge at 14,000 rpm for 30 sec and transfer 500 µL of the phage-containing supernatant to a new 1.5 mL tube.
4. Add 200 µL of 20 % (w/v) PEG/2.5 M NaCl. Invert several times to mix, and let stand for 10-20 min at room temperature.
5. Centrifuge at 14,000 rpm for 10 min at 4 °C and discard the supernatant, re-spin briefly and carefully pipet away and discard any remaining supernatant.
6. Suspend the pellet thoroughly in 100 µL of iodide buffer by vigorously tapping the tube. Add 250 µL of 100 % ethanol and incubate 10-20 min at room temperature (see **note 5**).
7. Spin at 14,000 rpm for 10 min at 4 °C, and discard the supernatant. Wash the pellet with 0.5 mL of chilled 70 % (v/v) ethanol, re-spin, discard the supernatant, and briefly dry the pellet.
8. Suspend the pellet in 30 µL of 1× TE buffer (see **note 6**) and quantify 1-2 µL in a NanoDrop spectrophotometer.

4. NOTES

1. TSB medium is a rich medium that is commonly used to culture members of the Enterobacteriaceae as well as for coliphage plaque assays. TSB is commercially available otherwise prepare as follows: to 1 L of distilled water add 17.0 g of Enzymatic Digest of Casein (Tryptone); 3.0 g of Enzymatic Digest of Soybean Meal (Soytone); 5.0 g of NaCl; 2.5 g of dipotassium phosphate (K_2HPO_4); and 2.5 g of Dextrose.
2. MTA is generally prepared with 0.6 % (w/v) agar but agar percentages between 0.4 % (w/v) and 0.7 % (w/v) can be used. Alternatively, MTA can be prepared with agarose instead of agar.
3. MTA can be stored at 47 °C if used within 1 day or at room temperature. Solid MTA can be melted using a water bath or a microwave oven.
4. Plates should be <1-3 days old, stored at 4 °C and have <200 plaques. Pick well-separated plaques. This will ensure that each plaque contains a single DNA sequence.

5. Short incubation at room temperature will preferentially precipitate single-stranded phage DNA, leaving most phage protein in solution.
6. The template can be suspended in sterile molecular biology grade water instead of TE if desired, but this is not recommended for long-term storage. In TE buffer the phage DNA should be stable indefinitely at -20 °C.

5. REFERENCES

1. Smith G.P. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* **228**:1315-1317.
2. Rakonjac J., Bennett N.J., Spagnuolo J., Gagic D. and Russel, M. (2011.) Filamentous Bacteriophage: biology, phage display and nanotechnology applications. *Curr Issue Mol Biol* **13**:51-76.
3. Sidhu S.S. (2000). Phage display in pharmaceutical biotechnology. *Curr Opin Biotechnol* **11**:610-616.
4. Pande J., Szewczyk M.M. and Grover A.K. (2010). Phage display: concept, innovations, applications and future. *Biotechnol Adv* **28**:849-858.
6. Martins I.M., Reis R.L. and Azevedo H.S. (2016). Phage display technology in biomaterials engineering: progress and opportunities for applications in regenerative medicine. *ACS Chem Biol* **11**:2962-2980.
7. Ebrahimizadeh W. and Rajabibazl M. (2014). Bacteriophage vehicles for phage display: biology, mechanism and application. *Curr Microbiol* **69**:109-120.
8. Deutscher S.L. (2011). Phage display in molecular imaging and diagnosis of cancer. *Chem Rev* **110**:3196-3211.
9. Christensen D.J. *et al*. (2001). Phage display for target-based antibacterial drug discovery. *Drug Discov Today* **6**:721-727.
10. Derda R., Musah S., Orner B.P., Klim J.R., Li L. and Kiessling L.L. (2010). High-throughput discovery of synthetic surfaces that support proliferation of pluripotent cells. *J Am Chem Soc* **132**:1289-1295.
11. Nobrega F.L., Ferreira D., Martins I.M., Suarez-Diez M., Azeredo J., Rodrigues L.R., Kluskens L.K. (2016). Screening and characterization of novel specific peptides targeting MDA-MB-231 claudin-low breast carcinoma by computer-aided phage display metodologies. *BMC Cancer* **18**:1-14.
12. Silva V.L., Ferreira D., Nobrega F.L., Martins I.M., Kluskens L.K., Rodrigues L.R. (2016). Selection of novel peptides homing the 4T1 cell line: exploring alternative targets for triple negative breast cancer. *PLoS One* **11**:e0161290.
13. Sergeeva A., Kolonin M.G., Molldrem J.J., Pasqualini R. and Arap W. (2006). Display technologies: application for the discovery of drug and gene delivery agents. *Adv Drug Del Rev* **58**:1622-1654.
14. Smothers J.F., Henikoff S. and Carter, P. (2002). Affinity selection from biological libraries. *Science* **298**:621-622.
15. Ferreira D., Martins I.M. (2016) "Artificial virus particles" *in* Bioinspired materials for medical applications 1st Edition. Ed. Lígia Rodrigues and Manuel Mota. WoodHead Publishing Series in Biomaterials, Elsevier, volume **121**, Chapter 15:427-450.
16. Giordano R.J., Cardo-Vila M., Lahdenranta J., Pasqualini R. and Arap, W. (2001). Biopanning and rapid analysis of selective interactive ligands. *Nat Med* **7**:1249-1253.

LIST OF PARTICIPANTS

Surname	Name	Institution	Country	E-Mail
Full course				
Balcão	Victor	University of Sorocaba	Brazil	victor.balcao@prof.uniso.br
Benedetti	Ilaria	Centro de Biología Molecular Severo Ochoa	Spain	i.benedetti@cbm.csic.es
Bonnain	Chelsea	University of South Florida	United States	cbonnain@mail.usf.edu
Carstens	Alexander Byth	Aarhus University	Denmark	aca@envs.au.dk
Cunningham	Alan	Teagasc	Ireland	alan.cunningham@teagasc.ie
Di Luca	Mariagrazia	Charité – Universitätsmedizin	Germany	mariagrazia.di-luca@charite.de
González Moreno	Mercedes	Charité - Universitätsmedizin	Germany	mercedes.gonzalez-moreno@charite.de
Hylling	Ole	Aarhus University	Denmark	ohy@envs.au.dk
Hosseini-doust	Zeinab	McMaster University	Canada	doust@mcmaster.ca
Janssens	Yorick	Ghent university	Belgium	yorick.janssens@ugent.be
Jutkina	Jekaterina	University of Gothenburg	Sweden	jekaterina.jutkina@gu.se
Kyrkou	Ifigeneia	Aarhus University	Denmark	ifigeneia.kyrkou@envs.au.dk
Lechuga	Ana	Centro de Biología Molecular Severo Ochoa	Spain	a.lechuga@cbm.csic.es
Lichvariková	Aneta	Comenius University Bratislava	Slovakia	lichvarikova.aneta@gmail.com
Lyons	Katriona	University College Cork/ Teagasc	Ireland	katriona.lyons@teagasc.ie
Majewska	Joanna	Polish Academy of Sciences	Poland	joanna.majewska@iitd.pan.wroc.pl
Malki	Kema	University of South Florida	United States	kema@mail.usf.edu
Mohaisen	Mohammed	University of Liverpool	United Kingdom	m.r.mohaisen@liv.ac.uk
Olsen	Nikoline S.	Aarhus University	Denmark	niso@envs.au.dk
Pennone	Vincenzo	Teagasc	Ireland	vincenzo.pennone@teagasc.ie
Ramirez	Luis	University Paris Sud	France	luis-maria.ramirez-chamorro@u-psud.fr
Rosíková	Kateřina	MB Pharma s. r. o.	Czech Republic	perzinovakaterina@gmail.com
Stukát	Gustaf	University of Gothenburg	Sweden	gustaf.stukat@vgregion.se
Stverakova	Dana	MB Pharma s.r.o., Masaryk University	Czech Republic	dana.stverakova@gmail.com
Tkacova	Adela	Slovak Academy of Sciences	Slovakia	adela.tkacova@savba.sk
Vidova	Barbora	Slovak Academy of Sciences	Slovakia	barbora.vidova@savba.sk
Wojtyna	Karolina	Polish Academy of Sciences	Poland	karolina.wojtyna@iitd.pan.wroc.pl
Zangelmi	Léo	Université Paris-Sud	France	leo.zangelmi@i2bc.paris-saclay.fr

Surname	Name	Institution	Country	E-Mail
Theoretical classes				
Garrido-Maestu	Alejandro	International Iberian Nanotechnology Laboratory	Portugal	alejandro.garrido@inl.int
Kaikano Ahmad	Adamu	National Veterinary Research Institute	Nigeria	karkafa@yahoo.co.uk
Markwitz	Paweł	University of Wroclaw	Poland	pawmarkwitz@gmail.com
Silva	Daniela	ALS Controlvet	Portugal	daniela.silva@alsglobal.com
Sosnowska	Ewelina	University of Wroclaw	Poland	ewelinasosnowska2@gmail.com
Wojtasik	Arkadiusz	Proteon Pharmaceuticals	Poland	awojtasik@proteonpharma.com