

## WELCOME MESSAGE

Dear participants,

It is my great pleasure to welcome you to the **4<sup>rd</sup> International hands-on PHAGE BIOTECHNOLOGY course**. This course is organized by the Bacteriophage Biotechnology Group (BBiG) at the University of Minho every two years since 2013. During the last years phage biotechnology has witnessed many scientific advances, and new emerging tools are having a great contribution in 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, characterization of phage-host interactions, genetic manipulation of phages and phage display.

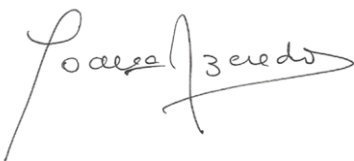
We are honored to have a speaker panel of excellent internationally recognized phage researchers that will deal with a broad range of subjects from phage ecology, structural analysis, phage-host interactions, omics and meta-omics phage characterization and phage engineering, to the application of phage 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 two social events: an outdoor “peddy-paper” team work activity and a dinner at the city center.

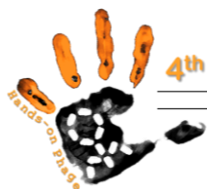
I wish to thank the lecturers who are contributing greatly to the high scientific standard of the course. I would also like to express my gratitude to all those who, through their dedicated efforts, have assisted us in the organization of this course.

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 2019



Joana Azeredo



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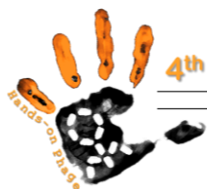
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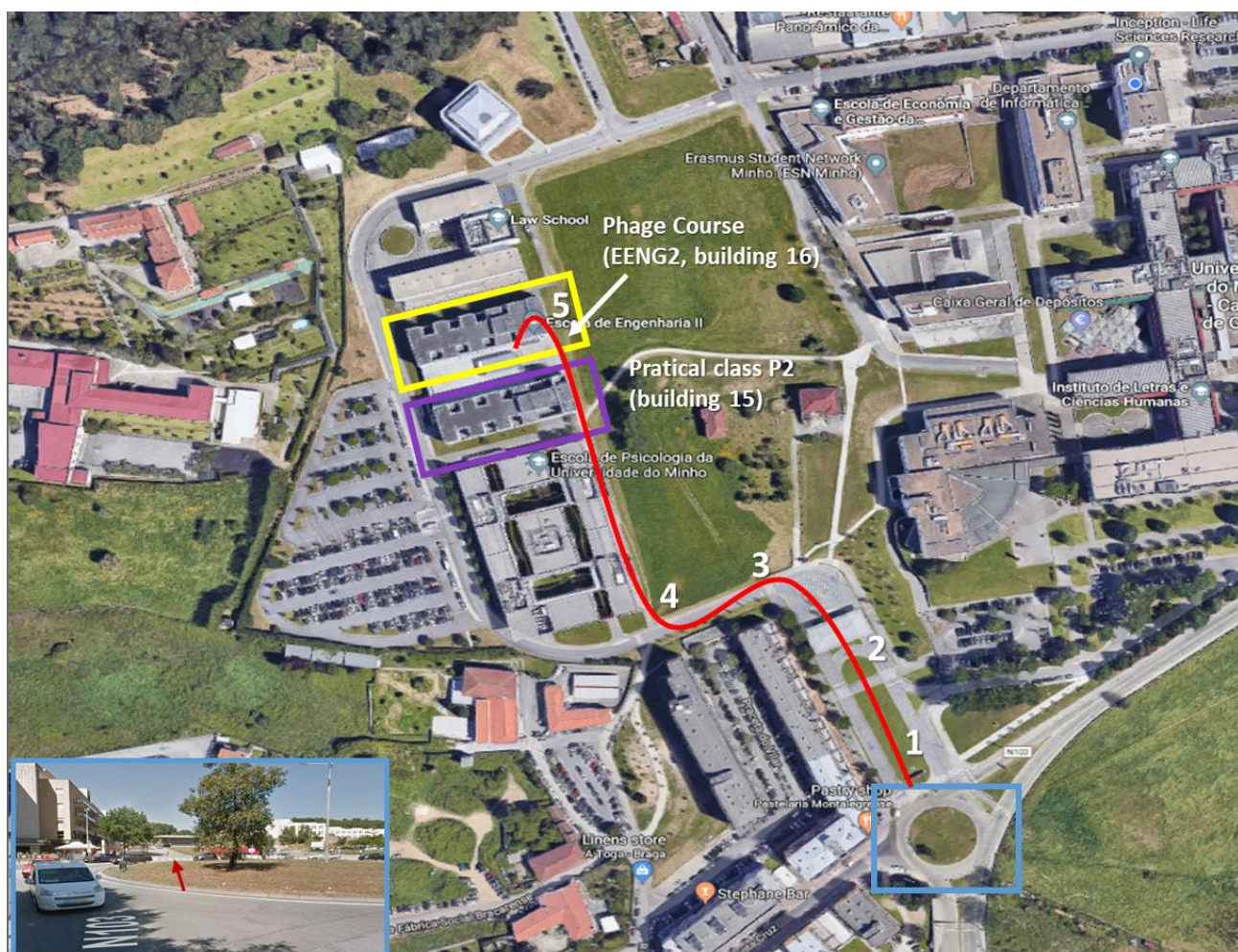


## GENERAL INFORMATION

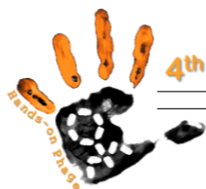
The 4<sup>th</sup> International hands-on PHAGE BIOTECHNOLOGY course will be held at EENG2, building 16, at Campus of Gualtar.

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 you reach the building 16 (yellow square).
5. The entrance of the building will be indicated by the course logo.

**Note:** Practical Session P2 will be held at building 15 (purple square).



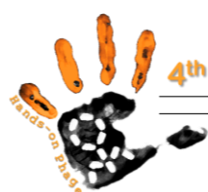




## SCIENTIFIC PROGRAM

### SCIENTIFIC PROGRAM AT A GLANCE

| Time          | THEORETICAL  |   | PRACTICAL                                  |  |
|---------------|--|---|--|--|
|               | 17 <sup>th</sup> June  | 18 <sup>th</sup> June   | 19 <sup>th</sup> June                      | 20 <sup>th</sup> June                      |
| 9:00 - 09:40  |  | Lecture 4 - Pascale Boulanger<br>"Structure and assembly of tailed bacteriophages"                                  |  |  |
| 09:40 - 10:20 |  | Lecture 5 - Danish Malik<br>"Scalable manufacturing and advanced encapsulation processes for bacteriophage therapy" | PRACTICAL (P1/P2)                          | PRACTICAL (P2/P1)                          |
| 10:20 - 10:30 |  |   |  |  |
| 10:30 - 11:00 |  | Coffee Break / Poster viewing   |  | Coffee Break                               |
| 11:00 - 11:40 |  | Lecture 6 - Dominic Sauvageau<br>"Host receptors for phage adsorption"  |  |  |
| 11:40 - 12:20 |  | Lecture 7 - Ivone Martins<br>"Phage display - innovative applications"  | PRACTICAL (P1/P2)                          | PRACTICAL (P2/P1)                          |
| 12:20 - 12:30 |  |   |  |  |
| 12:30 - 14:00 | Registration   | LUNCH / Poster viewing  |  | LUNCH                                      |
| 14:00 - 14:15 |  |   |  |  |
| 14:15 - 14:40 | Welcome session  |   |  |  |
| 14:40 - 15:20 | Lecture 1 - Marie-Agnès Petit<br>"Bacteriophage evolution"                       | Lecture 8 - Martin J Loessner<br>"Genetic manipulation of phages"   | PRACTICAL (P1/P2)                          | PRACTICAL (P2/P1)                          |
| 15:20 - 16:00 | Lecture 2 - Rob Lavigne<br>"Phage/host interaction: Omics approaches"            | Lecture 9 - Joana Azeredo<br>"Phage/Biofilm interaction: Challenges and Strategies"                                 |  | PRACTICAL (P4/P3)                          |
| 16:00 - 16:30 | Lecture 3 - Silvio B Santos<br>"Biotechnological applications of phage proteins" |   | Coffee break                               |  |
| 16:30 - 16:40 |  |   |  |  |
| 16:40 - 17:00 |  |   | Practical P1 / Phage Manipulation Workshop | Phage Manipulation Workshop / Practical P1 |
| 17:00 - 17:30 | SunSet   | Round Table   |  |  |
| 17:30 - 17:45 | Peddy-paper  | Group Photo   |  |  |
| 17:45 - 18:00 |  | Museum tour   |  | Farewell Party                             |
| 18:00 - 19:30 |  |   |  |  |
| 19:30 - 23:00 |  | DINNER  |  |  |



## DETAILED SCIENTIFIC PROGRAM

### June 17, Monday

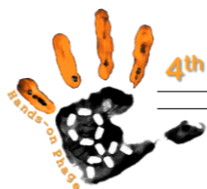
|             |                          |   |
|-------------|--------------------------|---|
| 12:30-14:15 | Arrival and Registration |   |
| 14:15-14:40 | Welcome Session          |   |
| 14:40-15:20 | Marie-Agnès Petit        | Bacteriophage evolution                         |
| 15:20-16:00 | Rob Lavigne              | Phage/host interaction: omics approaches        |
| 16:00-16:40 | Sílvia B. Santos         | Biotechnological applications of phage proteins |
| From 16:40  | Peddy-paper              |   |

### June 18, Tuesday

|             |                              |  |
|-------------|------------------------------|--|
| 09:00-09:40 | Pascale Boulanger            | Structure and assembly of tailed bacteriophages  |
| 09:40-10:20 | Danish Malik                 | Scalable manufacturing and advanced encapsulation processes for bacteriophage therapy                    |
| 10:20-11:00 | Coffee Break/Poster viewing  |  |
| 11:00-11:40 | Dominic Sauvageau            | Host receptors for phage adsorption  |
| 11:40-12:20 | Ivone M. Martins             | Phage display - Innovative applications  |
| 12:20-14:00 | Lunch Break / Poster viewing |  |
| 14:00-14:40 | Martin J. Loessner           | Bacteriophages 2.0: Synthetic biology and CRISPR-Cas genome editing enable tailor-made phage application |
| 14:40-15:20 | Joana Azeredo                | Phage/biofilm interaction: Challenges and strategies   |
| 15:20-16:00 | Jean-Paul Pirnay             | Phage therapy in the Brussels military hospital  |
| 16:00-16:30 | Coffee Break                 |  |
| 16:30-17:45 | Round Table                  |  |
| 17:45-18:00 | Group Photo                  |  |
| 18:00-19:30 | Museum tour                  |  |
| From 19:30  | Course Dinner                |  |

### June 19, Wednesday

|             |                                     |  |
|-------------|-------------------------------------|--|
| 09:00-10:30 | Practical 1 (P1) - Groups 1-4       | Bacteriophage isolation, production and purification |
|             | Practical 2 (P2) - Groups 5-8       | Bacteriophage genome annotation                      |
| 10:30-11:00 | Coffee Break                        |  |
| 11:00-12:30 | Practical 1 (P1) cont. - Groups 1-4 |  |
|             | Practical 1 (P2) cont. - Groups 5-8 |  |
| 12:30-14:00 | Lunch Break                         |  |
| 14:00-16:00 | Practical 1 (P1) cont. - Groups 1-4 |  |
|             | Practical 2 (P2) cont. - Groups 5-8 |  |
| 16:00-16:30 | Coffee Break                        |  |
| 16:30-18:00 | Practical 1 (P1) cont. - Groups 1-4 |  |
|             | Workshop - Groups 5-8               | How to engineer phage genomes?                       |



## June 20, Thursday

|             |                                     |  |
|-------------|-------------------------------------|--|
| 09:00-10:30 | Practical 1 (P1) - Groups 5-8       | Bacteriophage genome annotation                      |
|             | Practical 2 (P2) - Groups 1-4       | Bacteriophage isolation, production and purification |
| 10:30-11:00 | Coffee Break                        |  |
| 11:00-12:30 | Practical 1 (P1) cont. - Groups 5-8 |  |
|             | Practical 2 (P2) cont. - Groups 1-4 |  |
| 12:30-14:00 | Lunch Break                         |  |
| 14:00-16:00 | Practical 1 (P1) cont. - Groups 5-8 |  |
|             | Practical 2 (P2) cont. - Groups 1-4 |  |
| 16:00-16:30 | Coffee Break                        |  |
| 16:30-18:00 | Practical 1 (P1) cont. - Groups 5-8 |  |
|             | Workshop - Groups 1-4               |  |

## June 21, Friday

|             |                                     |   |
|-------------|-------------------------------------|---|
| 09:00-10:30 | Practical 3 (P3) - Groups 1-4       | Phage display technology                  |
|             | Practical 4 (P4) - Groups 5-8       | Monitoring bacteriophage/host interaction |
| 10:30-11:00 | Coffee Break                        |   |
| 11:00-12:30 | Practical 3 (P3) cont. - Groups 1-4 |   |
|             | Practical 4 (P4) cont. - Groups 5-8 |   |
| 12:30-14:00 | Lunch Break                         |   |
| 14:00-17:00 | Practical 3 (P3) - Groups 5-8       |   |
|             | Practical 4 (P4) - Groups 1-4       |   |
| 17:00-17:30 | Farewell Party                      |   |



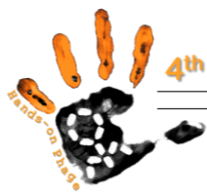
# LECTURE ABSTRACTS

JUNE 17-21, 2019 | BRAGA-PORTUGAL



4<sup>th</sup> **International hands-on**  
**PHAGE** BIOTECHNOLOGY **COURSE**





## Bacteriophage evolution

Marie-Agnès Petit

INRA, Micalis Institute, France

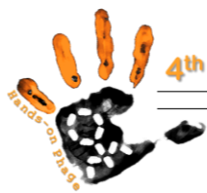
[marie-agnes.petit@inra.fr](mailto:marie-agnes.petit@inra.fr)

Bacteriophages have probably been on earth for as long as bacteria, they have therefore a long history of co-evolving with, and adapting to them. For example, they evolve by mutating the genes of their host recognition protein(s), to counteract the bacterial mutations changing the bacterial receptor. They can also mutate the region targeted by CRISPR spacers, or capture new genes, etc... At the root of this long lasting evolution, bacteriophages have peculiar molecular means permitting them to mutate and to recombine at frequencies higher than bacteria.

In the first part of this lecture, the various mechanisms leading to a higher mutation rate will be presented, with a particular emphasis on ongoing research in the field of mismatch repair. This process permits bacteria to correct wrong nucleotide insertions generated by DNA polymerases. In *Escherichia coli*, it is based on mismatch recognition, by the bacterial protein MutS, coordinated with recognition of the newly replicated strand, by the consecutive actions of Dam and MutH, and the molecular match maker MutL between MutS and MutH. In the absence of mismatch repair activity, ie when genes *mutL* or *mutS* are mutated for instance, mutation frequencies in the bacterial chromosome, per base and per generation, increase by a factor of 100. Interestingly, the same *mutL* or *mutS* mutations have much less effects on the mutation frequencies of bacteriophage genomes. Experiments to better understand how phages escape mismatch repair will be presented.

In the second part of the lecture, the properties of phage encoded homologous recombination proteins will be presented. Contrary to bacteria, who rely essentially on the ubiquitous RecA protein to recognize homologous DNA strands, bacteriophages encode a variety of genes to perform this function. At present, three large protein folds are known in phages to possess this strand pairing activity, Rad51-like, Rad52-like and Gp2.5-like proteins. Overall, among phage genomes of a size above 20kb, 60% have one of these pairing proteins. They pair DNA in a way distinct from RecA, and permit the exchange of DNA between sequences that are not strictly identical. This permits them to shuffle their genomes efficiently, and to capture new genes. Examples of such gene captures will be shown.

This survey of phage evolution mechanisms shows that phages have tuned their mutagenesis and recombination capacity slightly above the standards of the bacterial world, while still maintaining a certain genetic stability. Indeed, these higher frequencies are compensated by the high number of genome progeny (20-200 copies) per replication cycle, contrary to the bacterial world where only two chromosomes are produced at each replication cycle.



## Phage/host interaction: omics approaches

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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 transcription 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.



## Biotechnological applications of phage proteins

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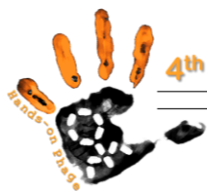
Bacteriophages have evolved for billions of years, developing a powerful protein armamentarium that enables them to recognize, infect and kill bacteria in a very efficient way. Bacteriophages encode many distinct proteins for the successful infection of a bacterial host. Each protein plays a specific role in the phage replication cycle, from host recognition, through takeover of the host machinery, and up to cell lysis for progeny release.

The global threat of antibiotic resistance has driven (and still is) research on the use of bacteriophages and their proteins to control multidrug resistant bacteria. Such interest, coupled with the recent progress in sequencing technologies, DNA manipulation and synthetic biology approaches, resulted in an increasing wealth of knowledge on the potential biotechnological application of phages. Phage-encoded proteins are now being explored in health, industrial, food, and agricultural settings, for purposes like detection and typing of bacteria; as vehicles for drug delivery; and for vaccine development. Many applications have been envisioned (which are not limited to bacteria control) and this is only considering the low percentage of phage proteins of known function. Research on this field is now progressing quickly and as the roles of these proteins are being revealed, more biotechnological applications can be anticipated.

Here I will overview the most recent progress reported on the use of phage-encoded proteins and highlight their most innovative uses, illustrating the biotechnological potential held by phage proteomes<sup>1</sup>.

### References

[1] Santos, Sílvia B et al. (2018), Trends in Biotechnology, 36(9), 966-984



## Structure and assembly of tailed bacteriophages

Pascale Boulanger

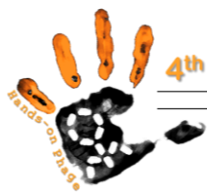
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[pascale.boulanger@i2bc.paris-saclay.fr](mailto:pascale.boulanger@i2bc.paris-saclay.fr)

Research into elucidating the assembly pathway and detailed structure of the tailed bacteriophages is essential for a better understanding of the interactions between phages and their host bacteria and to engineer specific phages for the development of new phage therapy strategies. Tailed bacteriophages are both sturdy nucleoprotein complexes that protect the viral genome from environmental insult and metastable structures that recognize a specific host receptor and efficiently deliver the viral genome to the bacterial cell for their replication. Their formation involves specific protein-protein and protein-nucleic acid interactions, as well as large conformational changes of assembly precursors. The sequence of events and molecular mechanisms of phage assembly can be elucidated by combining a large variety of methods, including mutational, biochemical and biophysical analyses, together with X-ray crystallography, NMR and cryo-electron microscopy.

All tailed bacteriophage consist of an icosahedral capsid containing a tightly packed dsDNA and a tail that recognizes the host cell, perforate its wall and serves as a pipe to deliver the viral genome into the host cytoplasm. In this lecture, several phage models for which a significant amount of knowledge has been accumulated will be used to illustrate the fascinating structural properties of these nanomachines : i) How the regulated stepwise assembly process of the capsid yields a robust genome container? ii) How conformational changes of the tail components ensure efficient propagation of the host-binding signaling from the Receptor Binding Proteins located at the tail tip to the capsid, for the release and delivery of the viral DNA?

These studies reveal that despite great genetic diversity, there are extensive structural similarities in the phage protein components and overall architecture. We expect that the recent advances in structural biology and in particular in cryo-electron microscopy instrumentation will made it possible to obtain atomic resolution structures of new phage particles, that in combination with phage genome analysis, will provide new insights in the phage-host interactions.



## Scalable manufacturing and advanced encapsulation processes for bacteriophage therapy

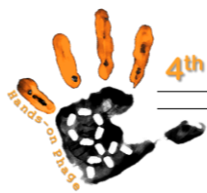
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Against a backdrop of global antibiotic resistance and increasing awareness of the importance of the human microbiota, there has been resurgent interest in the potential use of bacteriophages for therapeutic purposes, known as phage therapy. Recently concluded phage therapy clinical trials including the Nestlé trial to treat *Escherichia coli* associated diarrhea in children and the PhagoBurn effort by Pherecydes Pharma to treat *Pseudomonas aeruginosa* infections in burn wounds have highlighted major manufacturing, formulation and delivery challenges that need to be urgently addressed. Flexible modern scalable production approaches are needed to supply clinicians with phage products of suitable quality and stability to treat patients under compassionate use and for new clinical trials in order to demonstrate the considerable potential of bacteriophage therapy.

Targeted delivery and controlled release of high titres of purified phages at the site of infection remains a significant challenge. Oral delivery of unformulated phages could potentially result in inactivation of phages and reduction in phage titre upon exposure to gastric acidity thereby compromising efficacy of phage therapy. Targeting multi-drug resistant infections caused by bacteria that lead an intracellular lifestyle is another challenging problem as free phages may not be able to access eukaryotic cells without artificial vectorization approaches. This talk will present outcomes of recent research from our group on developing scalable process technologies for phage manufacture covering both upstream phage amplification, downstream purification as well as micro- and nano-encapsulation approaches of bacteriophages to address the aforementioned challenges.



## Host receptors for phage adsorption

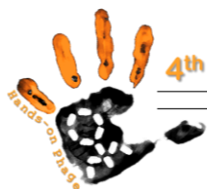
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The adsorption of a phage to its host is, of course, a crucial step of the infection process. The interactions between host receptors and phage receptor binding proteins mediate host recognition – in many ways defining the host range of a phage –, play a crucial role in co-evolution and the rise of resistance, and directly impact infection dynamics. Because of the critical role of adsorption in phage infections, more and more attention has been placed on identifying host receptors and understanding the mechanisms of adsorption.

In this lecture, we will first undergo a short survey of different types of host receptors and highlight how they can influence host-phage interactions. We will then investigate how these interactions are translated into different adsorption mechanisms, which impact both the kinetics of phage adsorption and population dynamics during phage infections. We will then focus on how factors such as host physiology, phenotypes and environmental conditions can affect adsorption and host recognition. Finally, we will look at how variations in host receptors and phage receptor binding proteins can impact infection, population dynamics and the implementation of phage-based technologies, such as phage therapy and pathogen detection.



## Phage display - Innovative applications

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Phage display was described for the first time in 1985 by Professor George P. Smith when he inserted a specific peptide sequence into the coat protein gene of a bacteriophage (phage), with consequent expression on the outside of the phage<sup>1</sup>. Indeed, Professor Smith has been awarded in 2018 with the Nobel Prize of Chemistry by the discovery of this technology.

Phage display involves the expression of proteins, including antibodies, peptides, gene fragment- or cDNA-encoded proteins and mRNA, expanding the practical applications of the technology. The concept of the technique is the incorporation of a nucleotide sequence into the phage genome as a fusion to a gene encoding a coat protein. The foreigner genes are thus found inside the virion while the protein is displayed on its surface.

This technology has been originally used has a traditional combinatorial library screening technique as a powerful mean to identify peptides that function as cell-surface ligands. However, it has broadened its application using phages as a scaffold to display a known molecule for the development of biologically active molecules, new biomaterials, probes for sensing and imaging, target drug, gene delivery vehicles and vaccines design<sup>2,3</sup>.

In this lecture, practical aspects of the technology as well as some applications will be discussed. Moreover, the major outcomes of the work developed in our research group using phage display to identify cell receptors on osteoarthritic cells and in cancer cells, and using phages as a tool to diagnose Alzheimer's disease, will be presented<sup>4-6</sup>.

### References

- [1] Smith, George P (1985), *Science*, 228, 1315-1317.
- [2] Martins, Ivone M et al. (2016), *ACS Chemical Biology*, 11, 2962-2980.
- [3] Ferreira, Débora et al (2016), in *Bioinspired materials for medical applications 1st Edition*, Elsevier, volume 121, Chapter 15:427-450.
- [4] Nobrega, Franklin et al. (2016), *BMC Cancer*, 18, 1-14.
- [5] Silva, Vera et al. (2016), *PLoS One*, 11, e0161290.
- [6] Ferreira, Débora et al. (2019), *Scientific Reports*, 9, 3958.



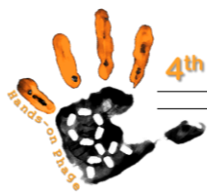
## Bacteriophages 2.0: Synthetic biology and CRISPR-Cas genome editing enable tailor-made phage application

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The incredible host specificity and inherent antimicrobial activity of bacteriophages is the basis for many diagnostic and antimicrobial and/or therapeutic applications, in agriculture, food safety, and medical microbiology. Examples include the use of phage-encoded affinity proteins for specific labeling, immobilization and rapid diagnostics of target cells such as *Listeria*, *Salmonella* and *Staphylococcus*, and the specific targeting and killing of bacterial cells by both intact phage and phage-encoded peptidoglycan hydrolases. However, application of phages is often restricted by several factors, such as narrow host ranges, genetic determinants encoding undesired factors and determinants, the ability for generalized transduction, and of course development of resistance against phage infection. To unleash the full potential of bacteriophages in therapy and biotechnology, genome editing is the way forward. Towards this aim, we developed new platform technologies to modify and optimize phage genomes. First, we developed a unique strategy employing cell wall-deficient bacterial L-form cells for bottom-up rebooting of tailor-made, fully synthetic virus genomes. This allows creation and realization of designer phages within 6-10 days, and is applicable to a wide range of phages infecting various pathogenic host bacteria. We found that synthesis of circular genomes offers several advantages, including a much better genome uptake and rebooting efficiency in the wall-deficient primary host cells. The only limit in this approach is the total size of the phage genome, which currently is restricted to approx. 80 kb. In addition, we identified the first fully functional CRISPR/Cas system within the genus *Listeria*, and used it to develop a molecular toolbox for rapid and efficient editing of the very large, non-integrating phage genomes. Altogether, these new approaches enable custom-made design, conversion and "payload-arming" of bacteriophages, and provide extremely useful tools for their application in phage-based therapy, biocontrol, and diagnostics of bacterial pathogens.



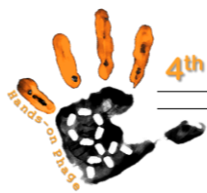
## Phage/biofilm Interaction: Challenges and strategies

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The intricate heterogeneous structure of biofilms confers to bacteria an important survival strategy in times of adversities even against their natural predators – the phages. In these structures, phages and cells establish a complex relationship to guarantee the long term survival of the progeny of both entities. Theoretically, the close proximity of cells within the biofilm structure could enhance phage-host interaction and facilitate phage infection. Conversely, the biofilm structure and composition as well as the physiological state of the biofilm cells may be an obstacle to phage infection. Nonetheless, phages have developed mechanisms to overcome biofilm barriers in a natural evolutionary prey-predator model. A thorough characterisation of biofilm/phage interaction and the identification of the weak aspects of biofilms and the strong features of phages are thus important to develop efficient phage-based biofilm control strategies. In this presentation studies involving the use of phages for the treatment or prevention of bacterial biofilms will be presented highlighting the biofilm features that difficult phage infection and the phage characteristics that enhances biofilm control. Also, some strategies based on combined therapies that can be used to enhance phage therapy against infectious biofilms will be presented.



## Phage therapy in the Brussels military hospital

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In 2003, a first phage therapy related study proposal was submitted to the R&D department of Belgian Defense. It was dismissed as mere “science fiction” with a score of 8/20. Fifteen years later, however, phage therapy has become commonplace in the Queen Astrid military hospital (QAMH). Phage therapy research in the QAMH encompasses diverse aspects:

- i) The isolation, selection, characterization and production (in cleanrooms) of therapeutic phages active against clinically important pathogens such as *Acinetobacter baumannii* (PMID: 25111143), *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Escherichia coli*, including the O104:H4 strain from the 2011 foodborne EAHEC outbreak in Germany (PMID: 23285164).
- ii) Clinical trials:
  - A small clinical safety study (PMID: 25356373): 10 applications of phage cocktail BFC 1 (PMID: 19300511), active against *P. aeruginosa* and *S. aureus*, in burn wound infections.
  - PhagoBurn ([www.phagoburn.eu](http://www.phagoburn.eu)), funded by the European Commission: Evaluating phage therapy for the treatment of burn wounds, infected with *P. aeruginosa*, through a randomized controlled trial (PMID: 30292481).
- iii) Study of the bacterium-phage (host-parasite) relationship, with an emphasis on bacterial phage resistance evolution and the development of adequate treatment protocols (PMID: 22660719, PMID: 26476097).
- iv) Under the umbrella of article 37 (unproven interventions) of World Medical Association’s “Declaration of Helsinki,” a number of patients with multidrug resistant infections were treated with phages in the Brussels military hospital (PMID: 28583189, PMID: 30884879).
- v) Elaboration of a dedicated regulatory framework for phage therapy, involving magistral phage preparations and including realistic production and QC/QA regimens (PMID: 21063753, PMID: 25585954, PMID: 29415431).

This presentation will give an overview of the evolution of phage therapy from “science fiction” to established therapy in the QAMH, and in Belgium in general.





# 4<sup>th</sup> International hands-on PHAGE BIOTECHNOLOGY course



## POSTER ABSTRACTS

JUNE 17-21, 2019 | BRAGA-PORTUGAL



## Genomic characterization and biodiversity of 37 coliphages in the intestine of poultry

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Bacteriophages (phages) are viruses that have the ability to specifically infect and kill bacteria. They are the most abundant biological entities on Earth and play essential roles in microbial ecological processes, including driving the diversity of the bacterial community, as well as in therapeutic and industrial applications (Clokier et al. 2011; Hatfull 2015). Phages are classified into families and subfamilies based on their host range and physical characteristics of the free virion, i.e. size, structure, morphology, and overall similarity of fully sequenced genomes and/or selected homologous conserved “marker genes” (Ackermann, 2009; Rohwer and Edwards, 2002). Despite the great importance of phages’ ubiquitous presence, little is known on the nature and extent of phage diversity of specific phages in specific ecosystems, and accordingly, phage genomic data constitute a large unexplored reservoir of genetic information, with numerous of genes whose function remains to be predicted (Grose and Casjens 2014; Hatfull 2015; Pope et al. 2015).

To better understand the *Escherichia coli*-infecting phage (coliphage) diversity in the intestines of poultry, we isolated and sequenced 37 coliphages from poultry faeces randomly collected from 27 poultry farms in Belgium. Phage isolation was performed with enrichment using an *E. coli* indicator strain K514 and the double-layer agar technique. The coliphage genomes ranged between 51,031-171,370 base pairs (bp), with a GC% content between 35.5-46.4%. A total of 72-275 coding sequences (CDSs) were identified for each phage using the Rapid Annotation using Subsystem Technology (RAST) server and the SEED viewer (<http://rast.nmpdr.org>). A phylogenetic comparison was performed using four genes; phage terminase large subunit, phage portal protein, capsid and scaffolding proteins, and the exonucleases. A maximum likelihood tree was constructed and included 13 reference coliphage genomes. The terminase large subunit analysis resulted in five distinct clusters: A-E, and four subclusters: A1, A2, D1 and D2. Clusters A, B, C, D and E comprised 20, 5, 5, 6 and 1 coliphage(s), respectively. The phylogenetic trees based on the three remaining gene groups (portal protein, capsid and scaffolding proteins, and exonucleases) resulted in a similar pattern, only with minor differences. These included additional duplicate or triplicate subclusters, comprised of the phages from cluster B, A or D subclusters. Based on the phylogenetic analysis, all phages were predicted to belong either to the *Siphoviridae* or *Myoviridae* family. Moreover, the phylogenetic analysis showed cluster formation according to phage family and subfamily, including, *Siphoviridae* > *Tunavirinae* (Cluster A and B), *Siphoviridae* > *Tequintavirus* (Cluster E), *Myoviridae* > *Ounavirinae* (Cluster C) and *Myoviridae* > *Tevenvirinae* (Cluster D). The phage classification (family) was confirmed by transmission electron microscopy.

The study provides a clearer understanding of the coliphage diversity in the intestines of poultry. However, one should be aware of possible biases as the phages were isolated on one strain and as such cannot be seen as the total coliphage diversity. In accordance with previous studies, these results suggest that determination of marker genes sequence type of newly isolated (coli)phages can provide a good preliminary indication of the cluster to which the phages belong. Furthermore, these genes can be used to investigate phage diversity and evolutionary relationships amongst phages (Dwivedi et al., 2012; Grose and Casjens, 2014).

## Bacteriophages and AMR: potential novel applications to control *Listeria* and *Campylobacter* antibiotic-resistant strains

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Phage application in food production and therapy is being considered a “new” potential tool in the fight against antibiotic resistant infections. In particular, the Italian Reference Laboratories for *Listeria monocytogenes* (NRL Lm) and *Campylobacter* (NRL C) are working for years in the isolation of environmental bacteriophages to be used as strategic means against bacterial contaminations in food and for animal therapy. In this work, we report our experiences in phages active against *Listeria monocytogenes* and *Campylobacter* antibiotic resistant strains. In particular, as results of *in vitro* and *in vivo* phage activities, we were also able to demonstrate the ability of phages to keep *Listeria* and *Campylobacter* antibiotic resistant strains at lower loads when compared with bacteria that were no phage-treated. Moreover, by analysing *Campylobacter* strains before and after phage treatment, we demonstrated that strains became resistant to phages. However, *Campylobacter* strains exhibiting resistance to ciprofloxacin, nalidixid acid and tetracycline before being phage treated resulted in apparent reversion to sensitivity to the respective antimicrobials after phage exposure.

## Structural insights and characterization of some mycobacteriophage derived LysB enzymes

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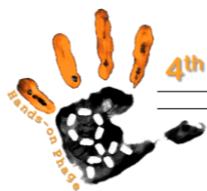
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*Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis (TB), is considered a health threat due to the highly emerged resistance rates<sup>1</sup>. In 2016, WHO reported 10.4 million new TB cases with 1.7 million mortalities out of which 40% were multidrug resistant TB (MDR-TB)<sup>2</sup>. The major characteristic feature of *Mtb* is their unique cell wall structure comprising peptidoglycan, arabinogalactan and mycolic acid (MA) layers<sup>3</sup>. MA is a long chain fatty acid (C60–C90) which is important for cell viability, imparting hydrophobicity and antibiotic resistance to *Mtb*<sup>4</sup>.

Mycobacteriophages possess two endolysins, Lysin A (LysA) a peptidoglycan hydrolase<sup>5</sup> and Lysin B (LysB), a lipolytic enzyme that cleaves the ester linkage of MA to the arabinogalactan-peptidoglycan layer<sup>6</sup>. We have screened genome sequences of 1700 mycobacteriophages mining for putative lysB genes using the available crystal structure of LysB-D29 as a template. Comparative study resulted in homology modeling of 30 LysB proteins different in their similarity percentage to LysB-D29. Sequence and 3D structural comparisons of those homology models to the 3D template of LysB-D29 were done in order to identify gaps in protein sequences and related differences in 3D structures. Subsequent docking studies of different p-nitrophenyl ligands (C4–C18) to the 3D models were performed to predict the potential enzymatic activity of each of the 3D homology models. Some LysB candidates were selected for cloning and expression in *E. coli* expression host. The esterase as well as the lipase activity of the purified enzymes were tested against p-nitrophenyl substrates (C4–C18), Tweens as well as the natural substrate mycolyl–arabinogalactanpeptidoglycan layer from *Mycobacterium smegmatis*. Moreover, inhibitory activity of the LysB enzymes was determined by viability assays against *M. smegmatis*.

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## Characterization of a unique *Bordetella bronchiseptica* vB\_BbrP\_BB8 bacteriophage

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*Bordetella bronchiseptica* is a Gram-negative pathogen that poses a threat to both humans and animals. Its co-infections with *Pasteurella multocida* result in multiple serious diseases. Both microorganisms are considered the crucial factors affecting the development of atrophic rhinitis in swine, thus triggering substantial financial losses in the agricultural industry. With an increasing number of multidrug resistant pathogens, there is a global need to develop effective alternative therapies. Phage therapy has long been considered an efficient weapon against pathogenic bacteria. In this work we present *B. bronchiseptica* vB\_BbrP\_BB8 lytic bacteriophage from the *Podoviridae* family. Analysis of the phage development showed that the phage is capable of infecting and killing *B. bronchiseptica* cells regardless of their growth stage. The genome of the vB\_BbrP\_BB8 phage was explored to evaluate the phage safety for use in therapy. The analysis of infection kinetics showed that after 1 minute 96% of the phage particles adsorbed to bacterial cells. Furthermore, we verified the phage potential to be used in the treatment of *B. bronchiseptica* infections. An assay involving honeycomb moth (*Galleria mellonella*) larvae indicated that over 90% of the larvae exposed to phage survived the bacterial infection, while all those in the control group died. We conclude that the phage is a promising candidate for use in phage therapy of *B. bronchiseptica* infections.



## Bacteriophages as a complementary tool of cleaning and disinfection

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*Salmonella* is one of the main serotypes involved on gastrointestinal diseases in Europe and most industrialized countries. Last data published by the EFSA, showed a total of 91.662 cases of salmonellosis in humans, being poultry products the main source of infection. The main serotypes involved are *S. Enteritidis*, *S. Typhimurium* and monophasic *Salmonella* Typhimurium variant. However, early this year, *S. Infantis* has increased, being the most prevalent in broiler production. In order to control this bacterium in poultry industry, in addition to the National Control Programs, biosecurity and cleansing and disinfections (C&D) protocols have been implemented. Nevertheless, the bacterium continues to be detected in some farms. For this reason, the implementation of innovative measures such as phage therapy is needed to control *Salmonella* at farm level. Bacteriophages or phages are virus that infect and replicate in prokaryotic cells, are specific of target bacteria, cost-effective and an environmentally friendly product. These characteristics makes phage's a promising complementary tool for *Salmonella* removal in those farms where disinfections procedures do not manage to eliminate the bacterium. In this context, the aim of this study was to assess the application of phages in an experimental farm as a supportive measure for C&D. Thus, 2 identical barns (A and B) were contaminated with *S. Infantis* with a  $10^8$  CFU/mL concentration. 24h after infection, all the facilities from 1 of the barns (A) were sprayed with the *S. Infantis*-phage ( $10^8$  PFU/mL). 48h after phage application, both barns were disinfected (A and B). 24h after disinfection, a total of 29 samples from the facilities were collected per barns (A and B). To assess the presence of *Salmonella*, samples were analysed according to ISO 6579:2017 (recommendations annex D). The results of this study showed that the 38% of samples analysed from the barn where no phage was applied (B) were positive, unlike barn 2, where only the 20% were positive (A). The results obtained in this study evidence that phages could be a complementary measure to C&D since it has been observed fewer positive samples to *Salmonella* in the barn where phage was applied.



## BoNT phage instability in *Clostridium botulinum* Group III

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*Clostridium botulinum* Group III is the main organism responsible for animal botulism, a deadly paralytic disease caused by botulinum neurotoxins (BoNTs) types C, D, and their mosaic forms C/D and D/C. The related neurotoxin gene loci are carried on large bacteriophages of the *Siphoviridae* family. These phages have a pseudolysogenic relationship with their bacterial hosts, thus they do not integrate into the chromosome. While large botulism outbreaks are reported every year and they cause substantial economic losses in affected farms, the biology of the BoNT phages remains poorly investigated. The BoNT phages are unstable and therefore easily lost during laboratory handling. Thus, only few BoNT phage sequences are available in public databases, and even fewer of them are of good quality (complete phage sequences available only for strains C-Stockholm producing BoNT/C and BKT015925 producing BoNT/C/D). No attempts have been made to compare the genetic composition of these phage sequences. Analysis of BoNT phages would help us to understand the phage biology and the interactions between the phages and Group III *C. botulinum*.

Here we investigated four *C. botulinum* Group III strains (C-Stockholm, BKT015925, BKT273, D-16868) for their BoNT phage instability. Strains BKT015925 and D-16868 lost the phage more readily than C-Stockholm and BKT273, suggesting BoNT phage instability to be strain dependent. Comparison of the related BoNT phage sequences using EDGAR (<https://edgar.computational.bio.uni-giessen.de>) allowed the identification of their core and accessory gene pools. Most of the identified phage core genes (60%) encode proteins of unknown function. The rest of the core elements encode structural and functional phage components, toxins, segregation-partitioning systems, and transcriptional regulators. The accessory phage genome contains transposons, CRISPR-elements, coding regions for restriction endonucleases, DNA-methyltransferases, toxin-antitoxin systems, and hypothetical proteins (representing 61% to 89% of the accessory content). The conserved core genome suggests that the various BoNT phages share a common ancestor. The diversity of the acquired accessory genetic contents, however, clearly suggests that the phages have further evolved in different environments. With 75% of the phage genetic content made of accessory elements, some of the acquired or lost genetic elements are likely to play a role in phage instability. Particularly, the *FtsZ* locus, which is involved in small replicon partition, is an interesting candidate to explain the differences in BoNT phage instability. This hypothesis remains to be explored.



## Functional impact of 12111phi prophage on *Streptococcus agalactiae* biofilm formation

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**Background:** *Streptococcus agalactiae* (GBS) is a leading cause of invasive infection in neonates. Infection mostly occurs following colonization of the new-born during delivery by inhalation or ingestion of GBS colonizing the maternal vaginal tract. Previous work (van der Mee-Marquet, CMI 2017) identified 6 groups (A to F) of temperate phages (prophages) integrated into the genome of 14 representative GBS strains. Prophages A (1) are inserted at four different sites, all located near bacterial genes involved in adaptation to environmental stress, and (2) carry four genes: *clpP*, *relB*, *yafQ* and *metK* that has been shown playing a role with biofilm formation, stress resistance and bacterial persistence (Hou, J. Basic Microbiol 2014; Yurong, Pathogens and disease 2014; Daimon, Journal of bacteriology 2015; Yadav, Microbial Pathogenesis 2012).

To investigate the functional impact of prophage A with GBS vaginal colonization, we studied the production of biofilm in a couple of isogenic GBS strains (e.g., the first being prophage free, the second being lysogenic for prophage A).

**Materials and methods:** A prophage-deleted derivative was first obtained from a GBS strain harbouring the prophage A 12111 following exposure to mitomycin. WGS was used to check if initial and deleted strains were isogenic. Then, the isogenic strains were studied for the production of biofilm using the crystal violet method (Borges, Antonie van Leeuwenhoek 2012; D'Urzo, AEM 2014) under three conditions: (1) after 24h incubation in Todd Hewit broth (TH) 1% glucose; (2) after 48h in TH 1 % glucose, and (3) after 24h in Roswell Park Memorial Institute (RPMI) glucose medium. Biofilm formation index (e.g. corresponding to optical density (OD) at 595nm of treated plate on OD of control plate) was used to compare the production of biofilm of the isogenic strains (Naves; Journal of Applied Microbiology 2008).

**Results:** Biofilm formation index it 1.5 with 24h incubation in TH glucose, 1.7 with 24 h incubation RPMI glucose medium and 2.3 with 48h incubation in TH glucose times higher for the lysogenic strain compared with the prophage free strain.

**Conclusion and perspectives:** Our data suggest a positive functional impact of t 12111phi prophage on GBS biofilm formation. To further explore the molecular mechanisms associated with the phenotypes observed, we currently construct prophagic *clpP*, gene-deleted mutants and *relB-metK* region deleted mutants.



## Stable isotope probing (SIP) for phage DNA

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Phages are ubiquitous in the environment and they are known to play a major role in the structuration of microbial communities<sup>1</sup>. Being able to identify phage hosts within microbial communities is thus of primary importance to achieve a deep understanding of ecosystem functioning. Very promising methods have recently emerged in this respect in molecular ecology<sup>2, 3</sup>. In addition, we are currently considering an original approach based on Stable Isotope Probing<sup>4</sup> to track phages infecting hosts involved in the assimilation of particular substrates. To our knowledge, the ability to establish a direct link between a virus and the metabolic activity of its host is specific to our proposed approach, and complementary to the other available methods.

To establish the proof-of-concept of the application of SIP to phage nucleic acids, we relied on a pure strain culture model, with *Escherichia coli* and T4 bacteriophage. T4 virions were produced by infection of *E. coli* cells grown on M9 minimal medium supplemented with either <sup>13</sup>C-glucose or unlabeled glucose as the sole carbon source. Each condition was performed in triplicate. According to the T4 DNA SIP profiles, the T4 DNA concentration peaks from the <sup>13</sup>C-glucose conditions were shifted towards higher densities compared to the peaks from the unlabeled-glucose conditions. This clearly evidenced the incorporation of <sup>13</sup>C in the DNA of T4 virions produced from the <sup>13</sup>C-glucose containing bacterial cell cultures.

These results reinforce our idea that SIP can be applied to identify host-virus relationships within complex ecosystems.

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## Identification and characterization of bacteriophages of bacteria inhabiting an extreme environment contaminated with heavy metals

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Viruses are major biological factors shaping structure and diversity of microbial communities. Prophages may provide multiple benefits to the host by the introduction of genes related to adaptation to the ecological niche.

The aim of this study is to discover novel host-phage systems and functional (culture-based) analysis of this host-parasite relationship. An environment selected to this analysis is Żłoty Stok gold and arsenic mine (Poland), characterized by low temperature, low oxygen and high concentration of heavy metals. This ecosystem is inhabited by unique groups of microorganisms adapted to its harsh conditions. Samples were collected from two areas: the bottom sediments (microbial mats) together with water and the mine walls (biofilm) of the end section of the Gertruda Adit, closed to visitors.

In this study biological samples obtained from the mine environment (biofilm, mats) were incubated for 7 days in the LB and R2A medium and next plated on agar. Water was filtrated, samples from the filter was collected and plated on LB or R2A agar. 16S rRNA gene sequence analysis allowed for taxonomic classification of bacteria to different genus: *Aeromonas*, *Bacillus*, *Flavobacterium*, *Janthinobacterium*, *Pseudomonas*, *Serratia* and *Shewanella*. In the next step, mitomycin C was used to induce active prophages from identified strains. Culturable bacteriophages were obtained using the concentrated environmental viral filtrate, bacterial isolates used as hosts and double layer agar plate method. DNA of these (pro)phages was isolated, sequenced, annotated and taxonomically classified. All novel phages belong to various families of *Caudovirales*.

In future research, we will focus on biological analysis of identified host-phage systems and selected genetic modules and also on metagenomic analysis of this environmental microbial community. The integration of metagenomic and genomic data will enable to study interaction between the viral and bacterial communities and their co-evolution.

## Characterization of the virome of *Paracoccus* spp. (*Alphaproteobacteria*) by combined *in silico* and *in vivo* approaches

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*Paracoccus* spp. (*Alphaproteobacteria*) are metabolically versatile bacteria that have been isolated from a wide range of environments in various geographical locations, including pristine and extreme environments as well as anthropogenically shaped ecological niches (e.g. contaminated soils and wastewater treatment plants). However, despite the wide knowledge about these bacteria, only 2 lytic phages - vB\_PmaS\_IMEP1 and Shpa - and a temperate phage - ΦPam-6 - all representing *Siphoviridae* family, are known. Hence, this study aimed to identify the unrecognized virome of *Paracoccus* spp. by combined *in silico* and *in vivo* approaches.

The collection of 16 *Paracoccus* strains representing different genera were screened for the presence for mitomycin C-inducible prophages. Induced prophages were isolated, their virions were visualized with transmission electron microscope and genomes were sequenced on Illumina MiSeq platform. Complete (9) and draft (55) genomes of *Paracoccus* spp. available in GenBank database were also screened for the presence of prophages using PhiSpy and manual inspection of each genome. All identified (pro)phages were manually (re)annotated and then compared one to another, as well as to all bacteriophages and viral contigs available in GenBank and IMG/VR databases, respectively. The comparison was performed on nucleotide and amino acid levels.

In this study 5 novel active temperate phages, originating from five different *Paracoccus* species, were identified and characterized (vB\_PbeS\_Pbe1, vB\_PkoS\_Pko1, vB\_PsuS\_Psu1, vB\_PthS\_Pthio1, vB\_PyeM\_Pye1), including the first (in the entire genus) representative of the *Myoviridae* family (vB\_PyeM\_Pye1). Moreover, in-depth bioinformatic analyzes of all available genomic sequences of *Paracoccus* spp. resulted in identification of 53 complete prophages within 29 strains, of which 13 were polylysogenic. This also led to identification of another *Myoviridae* and first (8) *Podoviridae* prophages. Manual reannotation of all 66 (pro)phages allowed to observe genes encoding proteins conferring metal resistance (11), toxin-antitoxin systems (10) and an extensive repertoire of DNA methyltransferases (88 within 53 (pro)phages). Most of DNA methyltransferase genes (59) were located between replication and packaging modules in phage genomes. Lastly, the complex comparative analyzes performed with the use of protein similarity network indicated that *Paracoccus* (pro)phages create a separate, distinct groups of bacteriophages.

Combination of *in silico* and *in vivo* approaches allowed for identification of 5 novel temperate phages and 53 prophages, providing the first insight into the diversity of *Paracoccus* spp. virome. It has showed the potential of thorough manual inspection of bacterial genomes in extending the knowledge on bacterial viruses, their diversity and potential influence on their hosts, as well as indication for new directions of phage studies.



## Extended characterization of three *Pseudomonas aeruginosa* phages and identification of host determinants required for their specific interaction

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Thermophilic composting harbors an impressive microbial richness and has proved a valuable source of information on novel bacteria and bacteriophages. Using *Pseudomonas aeruginosa* PA14 as host, we have previously isolated and characterized the genome of one phage from the *Siphoviridae* Yu-A like genus (ZC01) and two novel *Podoviridae* phages (ZC03 and ZC08) from Sao Paulo Zoo Park composting samples. These phages also showed promising antimicrobial activity *against P. aeruginosa*. Here we explore an extended host range and the structures involved in host-phage interactions, besides evaluation of phages stability and mature biofilm degradation effect. Infection assays of 70 different clinical and environmental *P. aeruginosa* isolates revealed a narrow host range for these phages. Phage susceptibility assays in *P. aeruginosa* PA14 mutants point to the type-IV pilus pilin (PilA) as the primary determinant for host spectrum, as the lack of lipopolysaccharide (LPS) do not affect PA14 susceptibility. The expression of pilin from PAO1, a *P. aeruginosa* strain resistant to the isolated phages, in a PA14 pilA null mutant, restored the twitching motility but not the phage-susceptibility. ZC01 and ZC03 were effective on mature biofilm (7 days) degradation, suggesting them as promising antimicrobial agents against *P. aeruginosa*. These studies provide insights into host-phage interactions and diversity between tailed phages. Comprehension of the adsorption step can enable our understanding of phage ecology and the development of phage-based technologies.



## Characterization of new *Campylobacter* phages isolated from pig feces

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*Campylobacter* is recognized as the leading cause of foodborne-illness in the European Union (EU) and worldwide. Pigs are known to be a natural reservoir of *Campylobacter* species with a prevalence of infection between 50 and 100%, being *Campylobacter coli* the most commonly detected followed by *Campylobacter jejuni*. Furthermore, the emergence of antibiotic resistant *Campylobacter* strains poses an additional threat to public health. In fact, the last EU summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food, highlighted high resistance levels to the harmonised set of antimicrobials in *C. coli* and *C. jejuni* isolates. Innovative effective strategies are, therefore, needed to reduce *Campylobacter* prevalence in primary production without use of antibiotics. The use of *Campylobacter* specific bacteriophages has been suggested as a promising alternative to reduce the prevalence of this pathogen within the farm-to-fork process. In this study, an enrichment method of Bolton broth and a mixture of 10 *Campylobacter* strains (five of *C. jejuni*, three *C. coli* and one strain each of *C. lari* and *C. fetus*) was used to isolate phages from pig feces samples. After purification and propagation, the genome size of the phages and their restriction fragment profiles were determined by PFGE and conventional electrophoresis respectively. The host range analysis of the new phage isolates was also carried out by inoculating 10  $\mu$ L spots of  $10^6$  PFU/ml suspensions onto 27 bacterial lawns including 11 *C. jejuni*, 13 *C. coli*, two *C. fetus* and one *C. lari*. Twelve phages were isolated and all of them, with a genome size of around 190 kb, were classified as group II campylophages or *Cp220virus*. The knowledge of the genome sizes of the phages allowed their genome restriction profiling, digesting their genomes by *Sma*I restriction endonuclease. Based on their host-range diversity, phages were classified into different lysis profiles. The use of these approaches to characterize the phages and analyze their lytic capacities allowed the selection of the most promising ones for their use as biocontrol agents. Further characterization of the selected phages is being carried out to determine their suitability for the future development of new campylophage-based products.



## Isolation and characterization of the lytic activity of two bacteriophage-derived proteins against *Campylobacter*

María Lavilla, Ibai Nafarrate, Amaia Lasagabaster  
AZTI, Spain

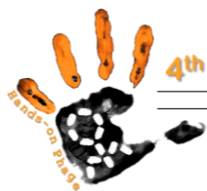
[mlavilla@azti.es](mailto:mlavilla@azti.es), [inafarrate@azti.es](mailto:inafarrate@azti.es), [alasa@azti.es](mailto:alasa@azti.es)

Most bacteriophages encode for lytic proteins whose function is to degrade the cell-wall of the bacterial host to release progeny virions. These proteins have a great potential as alternative to antibiotics or food preservatives. However, bacteriophage-derived lytic proteins have been proven to be more effective in Gram-positive bacteria than with Gram-negative bacteria.

In this work, two lytic proteins were isolated from two different *Campylobacter*-specific bacteriophages from AZTI's private collection, originally isolated from environmental sources. After isolation and purification, the lytic activity of these proteins at different concentrations was evaluated *in vitro* by spot testing against ten different *Campylobacter* strains. In addition, their effectivity after storage at three different temperatures (freezing: -20°C, refrigeration: 4°C, and room temperature: 22°C) was also characterized.

The two isolated proteins exhibited a narrow lytic spectrum, being effective against the 20% of tested *Campylobacter* strains. However, it is remarkable that the two proteins showed lytic activity against both *C. jejuni* and *C. coli* species at quite low concentrations (between 0.4 and 4 ppm, depending on the considered bacterial host). Moreover, the protein stock solutions (~0.4 mg/mL) maintained their activity for at least 6 weeks, even when stored at room temperature.

Our results suggest the potential of these proteins to be used as effective *Campylobacter*-specific biocontrol agents. However, further characterization (molecular characteristics, stability at other temperatures or pH storage conditions, host range against several *Campylobacter* strains of different origins, effectivity in foods, etc) is being carried out to determine their suitability for the future development of new anti-*Campylobacter* tools. Additionally, in our group, novel lytic proteins are being isolated looking for broader host range, higher anti-*Campylobacter* activity and other desired features.



## Coordination of cohabiting prophages in *Listeria monocytogenes*

Shai Ran Sapir, Tal Argov, Anna Pasechnek, Olga Stadnyuk, Lev Rabinovich, Gil Azulay,  
Ilya Borovok, Nadejda Sigal, Anat A. Herskovits  
Tel Aviv University, Israel

Most bacterial pathogens carry phage DNA within their genome. *Listeria monocytogenes* strain 10403S (*Lm*) carries two phage elements, one encodes the formation of infective virions (f10403S) and the other bacteriocins (monocins). Both phage elements encode for holin and endolysin proteins that have the capacity to lyse the bacteria under stress. During *Lm* infection of mammalian cells, the transcription of both phage elements is largely repressed. Notably, f10403S resides within the *comK* gene, interrupting its transcription. *comK* gene expression is important for *Lm* infection of mammalian cells. During macrophage cells infection, f10403S excises the chromosome, resulting in the formation of an intact *comK* gene, though virion production is blocked. In this work I present the characterization of a mechanism that synchronizes the two phage elements under SOS and intracellular conditions using a single protease, named here MpaR. MpaR is encoded in the monocin-phage element and its activation leads to the excision of f10403S, enabling the transcription of *comK* within mammalian cells.

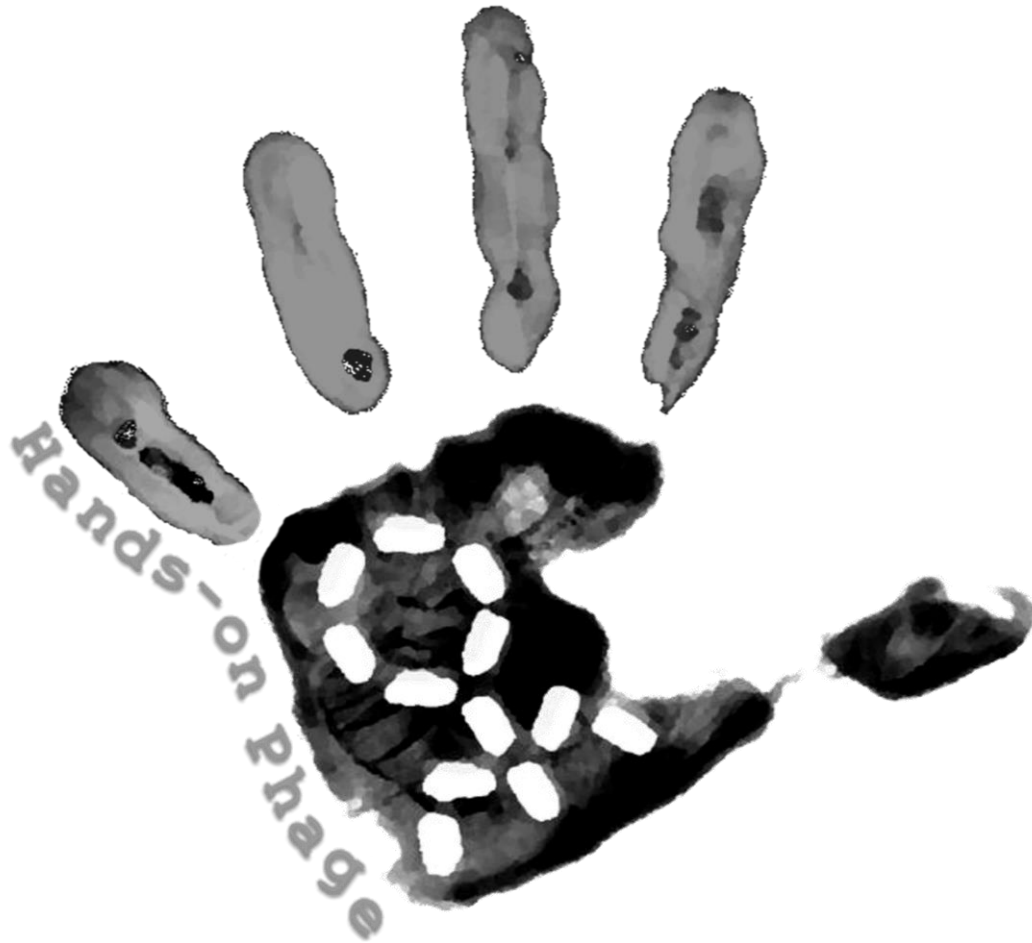
## Enhanced virulence synthetic bacteriophage consortium against *Ralstonia* using silicon nano-particle

Mohammadhossein Ravanbakhsh  
Utrecht University, Netherlands

*Ralstonia solanacearum* is a plant pathogenic bacteria caused bacterial wilt disease in the wide range of plant hosts. Bacteriophage therapy can be one of the promising approaches to control this pathogen. However, *R. solanacearum* got resistance to bacteriophage over bacteria-phage co-evolution. Our goal is to produce an enhanced virulence synthetic bacteriophage consortium against *Ralstonia*, by aiding silicon nano-particle. Our primary results show the combination of bacteriophage and nano-particle decrease the ability of bacteria evolve resistant to bacteriophage during the course of evolution. We will test the most promising phages, and nanoparticles combinations, in a full factorial design in vitro experiment. The final goal would be selecting the best bacteriophage and nanoparticle for greenhouse condition as prototype applications.

# WORKSHOP

JUNE 17-21, 2019 | BRAGA-PORTUGAL



4<sup>th</sup> **International hands-on**  
**PHAGE** BIOTECHNOLOGY **COURSE**



## How to engineer phage genomes?

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The latest advances in the synthetic biology field have enabled the development of new molecular biology tools that can be used to build specialized bacteriophages with new functionalities. Recently, phages have been engineered towards a wide range of applications including pathogen control and detection, targeted drug delivery, or even assembly of new materials<sup>1</sup>.

In this workshop, the strategies currently used to build synthetic phages will be addressed and a practical case study will be analyzed. In this practical case, the students will follow all the steps needed to perform a gene knock-out in a phage genome using one of the phage-engineering strategies that have been successfully applied in our lab: the yeast-based assembly platform<sup>2,3</sup>.

### References:

- [1] Costa AR et al. (2018), In: Methods in molecular biology, pp 285–300.
- [2] Pires DP, et al. (2016). Microbiology and Molecular Biology Reviews, 80:523–543.
- [3] Ando H, et al. (2015), Cell Systems, 1:187–196.



# PRATICAL SESSIONS

JUNE 17-21, 2019 | BRAGA-PORTUGAL



4<sup>th</sup> **International hands-on**  
**PHAGE** BIOTECHNOLOGY **COURSE**



## PRACTICAL SESSION P1

### Bacteriophage isolation, production and purification

Ana Oliveira\*, Alice Ferreira, Ergun Akturk, Henrique Ribeiro, Katarzyna Gembara, Maria Daniela Silva, Susana Costa

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#### 1. INTRODUCTION

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<sup>1-3</sup>. In this work we propose the isolation of different phages<sup>4-10</sup>, 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<sup>11,12</sup>. 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 in order 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.

#### Phage isolation

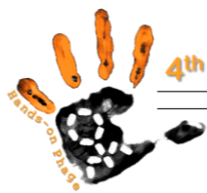
In order to discriminate different phages a positive sample will be serially diluted and plated. Discrete phage plaques will be observed, representing bacterial lysis caused by a single phage. Plaque characteristics are related to the type of bacteriophage, bacterial host characteristics as well as to other physical and chemical characteristics of the system in which the bacteriophage was produced<sup>13-15</sup>. Different phage plaques, if observed, will be segregated.

#### Phage production

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

#### Phage purification

After production, purification may ensure a pure and debris-free phage suspension that can be used for several applications. Detailed protocols for polyethyleneglycol (PEG 8000) precipitation and cesium chloride (CsCl) gradient ultracentrifugation are supplied as supporting material (Annex I).



## Phage characterization

To assess the infectious potential of phages for different applications, several biological characterization assays can be performed. Herein, we describe the two most common characterization procedures: i) lytic spectra determination and efficiency of plating to evaluate the phage host range and efficiency of infection, respectively; ii) one-step growth curve, to determine the phage latent period and burst size, to characterize phage fitness and potential to control the target host<sup>16-18</sup>.

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 between the individual restriction fragment length polymorphism (RFLP) patterns<sup>1,3</sup> or between patterns obtained by randomly amplified polymorphic DNA (RAPD)-PCR technique.

## 2. MATERIALS

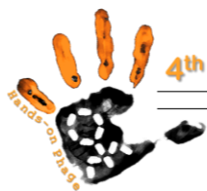
Prepare all solutions using distilled water. The medium used in the procedures described herein is Tryptic Soy Broth (TSB) (see **note 1**) but alternative medium can be used, depending on the bacterial species. Sterilize (autoclave at 121 °C for 15 min) all the solutions and material and store at room temperature or 4 °C for longer use.

### 2.1. Phage enrichment from collected samples

- Samples for phage isolation:
  - solid samples (e.g. sludge/soil)
  - liquid samples (e.g. sewage from wastewater)
- 50 mL Falcon tubes;
- TSB broth;
- Double strength TSB (2× TSB) broth;
- Overnight grown bacterial hosts;
- 100 mL and 500 mL bottles;
- 500 mL Erlenmeyer flasks;
- 0.2 µm PES filters;
- Syringes
- NaCl 0.9 % (w/v).

### 2.2. Preparation of bacterial lawns by pour-plate technique

- Overnight grown bacterial hosts;
- Agar plates (TSA) containing:
  - TSB with 1.2-1.5 % (w/v) agar;
  - Pour on Petri dishes after autoclaving;
- Molten Top-Agar (MTA\_TSB): TSB with 0.6 % (w/v) agar. Store accordingly (see **note 2**);
- 15 mL test tubes.



### 2.3. Phage isolation

- Overnight grown bacterial hosts;
- MTA\_TSB (47 °C);
- TSA plates;
- Sterile paper strips (approximately 1 cm × 5 cm);
- Sterile toothpicks;
- 15 mL Falcon tubes.

### 2.4. Phage production

- Overnight grown bacterial host;
- TSA plates;
- MTA\_TSB (47 °C);
- SM 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 of the buffer with HCl to 7.5. Then, to a 1 L bottle, add 5.8 g of NaCl, 2.0 g of MgSO<sub>4</sub>·7H<sub>2</sub>O and 50 mL of the prepared 1 M Tris-HCl (pH 7.5), and make up to 1 L with water. Autoclave (see **note 3**);
- 50 mL Falcon tubes;
- 250 mL Erlenmeyers flasks;
- 50, 100, 200 mL bottles;
- Sterile paper strips;
- 0.2 µm PES filters;
- Sterile toothpicks;
- Chloroform.

### 2.5. Phage titration

- TSA plates;
- MTA\_TSB (47 °C);
- SM buffer (see **note 3**);
- 1.5 mL Eppendorf tubes;
- 15 mL Falcon tubes.

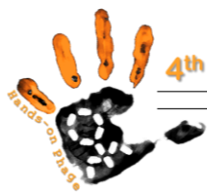
### 2.6. Phage biological characterization

- Bacterial culture in TSB;
- TSA plates;
- MTA\_TSB;
- SM buffer;
- 1.5 mL Eppendorf tubes;
- 15 mL Falcon tubes;
- 250 mL Erlenmeyer flasks.

### 2.7. Phage DNA extraction, quantification and quality assessment

#### DNA extraction

- Purified phage samples;
- Phage DNA extraction kit (see 3.7.1);
- Ultrapure water;
- Sterile 1.5 mL microcentrifuge tubes;



#### DNA quantification and quality assessment

- Phage DNA samples;
- Ultrapure water;
- Nanodrop;
- Agarose Gel electrophoresis system;
- Agarose;
- TAE 1X (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0);
- DNA ladder (e.g. 1 Kb Gene ruler);
- DNA Loading Dye (6X);
- Green Safe Stain;
- Equipment for agarose gel analysis.

#### RFLP

- Phage DNA samples;
- Restriction enzymes;
- Sterile 1.5 mL microcentrifuge tubes;
- Agarose Gel electrophoresis system (see below);
- Agarose;
- TAE 1X;
- DNA ladder;
- DNA Loading Dye (6X);
- Green Safe Stain;
- Equipment for agarose gel analysis.

#### RAPD-PCR

- Phage DNA samples;
- Primers, Master mix;
- Sterile 1.5 mL microcentrifuge tubes;
- Agarose Gel electrophoresis system (see below);
- Agarose;
- TAE 1X;
- DNA ladder (e.g 1Kb Gene Ruler);
- DNA Loading Dye (6X);
- Green Safe Stain;
- Thermocycler;
- Equipment for agarose gel analysis.

### 3. METHODS

All procedures will be performed 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”<sup>19</sup>.



### 3.1. Phage enrichment

1. Put 50 mL of the liquid samples in 250 mL sterile Erlenmeyers; For solid samples, add 50 mL of NaCl 0.9 % solution and 10 g of solid sample to 250 mL Erlenmeyers;
2. Add 50  $\mu$ L of overnight grown bacterial suspensions (at least 5 different non-lysogenic strains) (see **note 5**) and 50 mL of 2 $\times$  TSB to the Erlenmeyer 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 Falcon tubes and centrifuge (9,000  $\times$ g, 4 °C for 10 min);
5. Collect and filter (PES filter 0.2  $\mu$ m) the supernatant to sterile 50 mL Falcon tubes.

### 3.2. Preparation of bacterial lawns

1. Add 100  $\mu$ L 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. Phage isolation

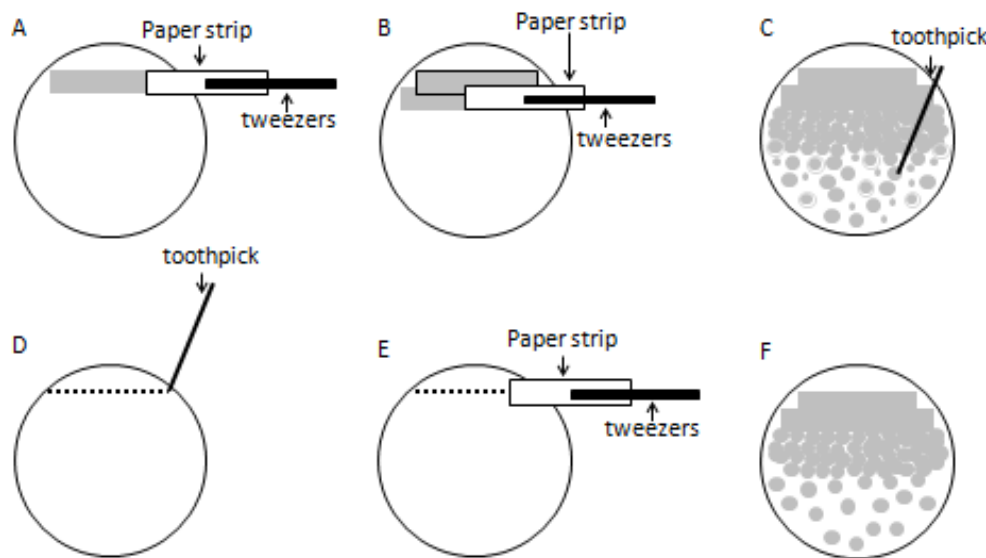
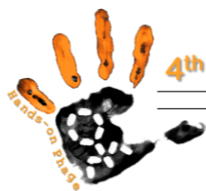
#### 3.3.1. Spot test verification of the enriched samples

1. Add 10  $\mu$ L 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 6**);
2. Let the plate stand until completely dried;
3. Incubate the plate overnight at the proper growth temperature;
4. Check for clear or turbid lysis zones indicative of the presence of phages (lysis zones can also be due to other factors like the presence of bacteriocins; to be sure that the observed phenomena is due to phage activity, you need to go to step 3.3.2).

#### 3.3.2. Phage 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 7**) in the bacteriophage 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 (**Figure 1A**);
3. Streak downwards, changing the paper strip after every streak, making certain that the paper strip touches the previous streak (**Figure 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 **Figure 1C**);
6. Pick different plaques with a toothpick and stick it several times (in a line) in an agar plate with a bacterial lawn prepared as described in 3.2. (**Figure 1D**);
7. Use sterile paper strips to streak the phages as described above (3.3.2.3) (**Figure 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 (**Figure 1F**) (see **note 8**);
9. Add 3-5 mL of SM buffer to each plate and incubate at 4 °C during 5-18 h at 50 – 90 rpm;
10. Collect the SM buffer to a falcon tube and add chloroform to a final concentration of 10%;
11. Centrifuge the solution (9,000  $\times$ g, 4 °C, and 10 min) to remove all bacteria;
12. Collect and filter (0.2  $\mu$ m) the supernatant;
13. Store at 4 °C until needed (phage stock).



**Figure. 1.** Isolation of bacteriophages. A) initial streak; B) streaking downwards on a bacterial lawn; C) isolated bacteriophage plaques and picking a single colony with a toothpick; D) making a puncture line with a toothpick; E) streaking with paper strips; F) morphologically identical plaques.

### 3.4. Phage production

#### 3.4.1. Phage production using the soft-agar overlay technique

1. Add 100  $\mu$ L of the bacterial culture grown overnight and 100  $\mu$ L of diluted phage ( $\sim 1 \times 10^5$  PFU/ mL) to TSA plates and mix gently.
2. Incubate for 10 min at the proper temperature to allow phages to adsorb to the host bacterium (this time period may vary with phage/host);
3. Add 3-5 mL of MTA\_TSB (47°C) and let it solidify;
4. Incubate overnight, without inverting, at the proper temperature;
5. Add 1-3 mL of SM buffer to the TSA plates and incubate overnight at 4 °C;
6. Transfer the SM buffer with the eluted phages to 50 mL Falcon tubes (see **note 9**);
7. Add chloroform to a final concentration of 10 %;
8. Centrifuge the solution (9,000  $\times$ g, 4 °C, and 10 min) to remove all bacteria and debris;
9. Carefully collect the supernatant, filter (0.2  $\mu$ m) and store at 4°C until further use.

#### 3.4.2. Phage production using a suspended bacterial culture

1. Grow bacteria in 100 mL Erlenmeyers, containing 25 mL of sterile TSB, until cells reach the mid-exponential phase ( $OD_{600}=0.4$ );
2. Dilute the phage stock solution to have a concentration of approximately  $1 \times 10^5$  PFU/mL (MOI approx.  $\leq 0.001$ ) (see **note 10**);
3. Add 1-5 mL of the diluted phage and 25 mL of the bacterial culture (from step 1) to a 250 mL Erlenmeyer;
4. Incubate at 37 °C, 120 rpm);
5. When the culture turbidity decreases (4-6 h), transfer to 50 mL Falcon tubes, add chloroform to a final concentration of 10 % and centrifuge (9,000  $\times$ g, 4 °C, and 10 min);
6. Filter-sterilize (0.2  $\mu$ m) and store at 4°C until needed.



### 3.5. Phage titration

#### 3.5.1. Enumeration using the small drop plaque assay

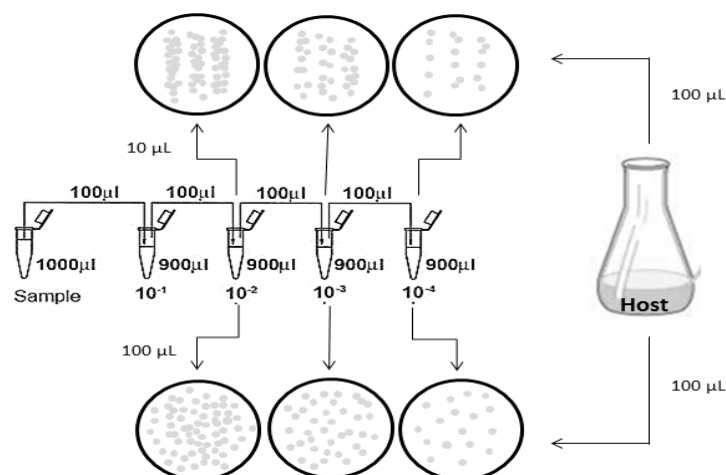
1. Prepare serial dilutions (1:10) in SM buffer of the phage stock in 1.5 mL Eppendorf tubes);
2. Add 100  $\mu$ L of bacterial culture grown overnight, and 3-5 mL of MTA\_TSB (47 °C) to a TSA plate and gently mix. Let the plates dry for 10 min;
3. Add a drop of 10  $\mu$ L of the dilution mixture onto a TSA plate, tilt the plates at 45 ° and then let them still;
4. After drying, incubate overnight at 37 °C;
5. Count the plaques formed in the drop of the dilution with 3-30 phage plaques (**Figure 2**);
6. Determine the titer of replicates according to Equation 1.

#### 3.5.2. Enumeration by double agar overlay

1. Using the prepared serial dilutions, add 100  $\mu$ L of phage solution, 100  $\mu$ L of bacterial culture grown overnight, and 3-5 mL of MTA\_ (47 °C) to a TSA plate and gently mix;
2. Let the plates dry for 10 min and incubate at 37 °C;
3. Count the phage plaques in the dilution which resulted in 20-200 plaques (**Figure 2**);
4. Determine the titer of replicates according to Equation 1.

Equation 1:

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



**Figure 2** - Phage enumeration using the small drop plaque assay (upper panel) and by double agar overlay (lower panel).



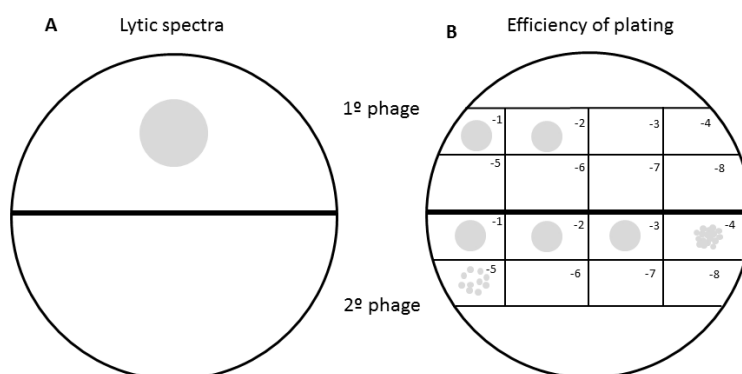
### 3.6. Phage Characterization

#### 3.6.1. Lytic spectra

1. Grow bacterial strains in 100 mL Erlenmeyer flasks, containing 25 mL of TSB, until cells reach exponential phase ( $OD_{600}=0.4$ );
2. Poor 100  $\mu$ L of bacteria suspensions into TSA plates, stir with 3-5 mL of MTA\_TSB (47 °C) to form an uniform lawn, and let it dry for 10 min;
3. Spot, individually, 10  $\mu$ L of each undiluted phage sample on the lawns and incubate overnight at 37 °C;
4. The presence of areas with bacterial growth inhibition was indicative of host susceptibility to the phage (**Figure 3A**).

#### 3.6.2. Efficiency of plating assay

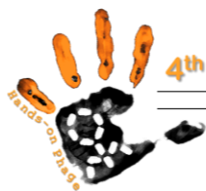
1. Grow bacterial strains in 100 mL Erlenmeyer flasks, containing 25 mL of TSB, until cells reach exponential phase ( $OD_{600}=0.4$ );
2. Poor 100  $\mu$ L of bacteria suspensions into TSA plates, stir with 3-5 mL of MTA\_TSB (47 °C) to form an uniform lawn, and let it dry for 10 min;
3. Prepare serial dilutions (1:10) in SM buffer of the phage samples and spot, individually, 5  $\mu$ L of each phage dilution, into the lawn and incubate overnight at 37 °C;
4. Determine the efficiency of plating, i.e., the relative phage titer on a bacterial strain compared to the maximum titer observed;
5. Score high, moderate and low efficiency of plating when phage titer for each strain represent 100-10 %, 1-0.1 % and 0.01-0.001 %, respectively (**Figure 3B**). Consider lysis-from-without (LFW) when plaques appear only in higher dilutions, with no progression.



**Figure 3** – Lytic spectra and efficiency of plating graphic representations

#### 3.6.3. One-step Growth Curve (OSGC) parameters

1. Grow bacteria in 100 mL Erlenmeyer flasks, containing 25 mL of TSB, until cells reach mid-exponential phase ( $OD_{600}=0.4$ );
2. Centrifuge 10 mL of suspension at 7,000  $\times g$ , 5 min, 4 °C, and discard the supernatant;
3. Resuspend the pellet in 5 mL fresh media ( $OD_{600}$  of approx. 0.4;  $\approx 10^8$  CFU/mL);
4. Add 5 mL of phage with a titer of  $8 \times 10^5$  PFU/mL to have the desired MOI ( $\leq 0.001$ ) (**see note 10**);
5. Incubate with agitation for 5 min to allow phages to adsorb to the host cells;
6. Centrifuge the 10 mL culture at 7,000  $\times g$ , 5 min, 4 °C and discard the supernatant;



7. Resuspend the pellet in 10 mL fresh TSB medium;
8. Incubate at 37 °C under agitation (150-200 rpm);
9. Determine the phage titer immediately. This sample will represent time zero of the experiment. Repeat the titration every 10 min, until 60 min;
10. The OSGC is used 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 (**Figure 4**).

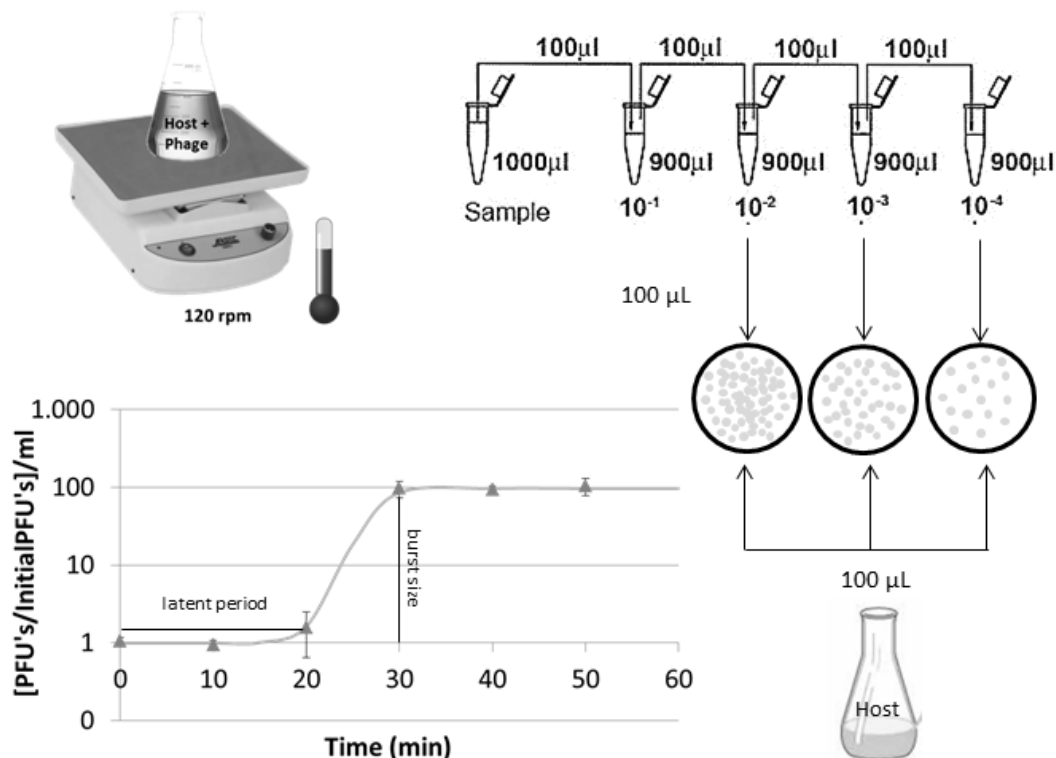


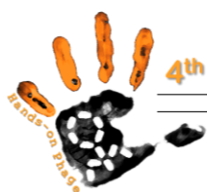
Figure 4 – One-step growth curve.

### 3.7. DNA extraction, quantification and quality assessment

#### 3.7.1. DNA extraction

There are several protocols for carrying out phage DNA extraction. Two different approaches are suggested – a phenol/chloroform classic DNA extraction (Annex II) or the use of commercial kits (previous treatment with DNase/RNase shall be included):

| 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            |



### 3.7.2. DNA quantification and quality assessment

#### Nanodrop

1. Place 1.5  $\mu$ L of ultrapure water onto the lower optic surface. Clean both optical surfaces with a wipe;
2. Perform a blank measurement with 1.5  $\mu$ L of DNA elution buffer. Clean both optical surfaces with a wipe;
3. Measure the nucleic acid samples by loading 1.5  $\mu$ L of each sample. Clean both optical surfaces with a wipe after the measurement of each sample;
4. After measurement, Nanodrop software will provide sample concentration, as well as 260/280 and 260/230 ratios (**see note 11**).

#### Agarose Gel Electrophoresis

1. Weigh the agarose (1 %, w/v), resuspend in TAE buffer and dissolve by heating in the microwave;
2. Cool the agarose solution and add the dye (0.006% (v/v)). Pour the gel and let dry;
3. Add the appropriate volume of 6x loading dye to the samples and load them in the wells, as well as the DNA ladder (5  $\mu$ L);
4. Run the gel at 100 Volts (about 1 h) in 1x TAE (**see note 12**);
5. Analyse the gel under UV light.

### 3.7.3 RFLP

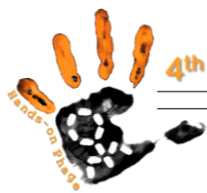
1. Digest around 3  $\mu$ g of genomic DNA with 10 Units of the selected restriction enzymes;
2. Incubate the samples at the suitable temperature (check the temperature and time recommended by the manufacturer);
3. Prepare a 0.6-0.8 % agarose gel in 1x TAE, and add the dye;
4. Add to each sample the appropriate volume of 6x loading dye to a final concentration of 1x and load them in the wells; add the DNA ladder;
5. Run the gel 2 h at 70 Volts in 1x TAE and analyse the gel under UV light.

### 3.7.4 RAPD-PCR

1. Prepare a PCR reaction with 10-50 ng of purified phage DNA per reaction, 1 U of a DNA polymerase, 0.2 mM dNTPs and 2 mM of each of these primers: P1 (5'-CCGCAGCCAA-3') and P2 (5'-AACGGGCAGA-3'). Reaction using Xpert Fast Master Mix (2X) (Grisp):

| Component                               | Volumes ( $\mu$ L) |
|---|--------------------|
| Xpert Fast Master Mix 2X DNA polymerase | 12.5               |
| Primer P1                               | 5                  |
| Primer P2                               | 5                  |
| Template DNA                            | Adjust to 10-50 ng |
| Water                                   | Up to 25 $\mu$ L   |

2. Use the following thermal cycling conditions 94 °C for 10 min; 40 cycles at 94 °C for 60 sec, 35 °C for 30 sec and 72 °C for 60 sec; a final extension step of 10 min at 72 °C.
3. Prepare a 0.8 % agarose gel in 1x TAE, and add the dye, load the samples with loading dye (final concentration 1x) in the wells and add the DNA ladder;
4. Run the gel 40-60 min at 90 Volts in 1x TAE and analyse the gel under UV light.

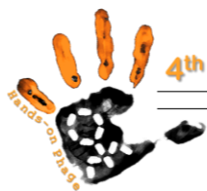


## 4. NOTES

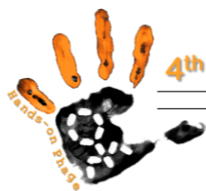
1. TSB medium is a rich medium that is commonly used to culture members of the Enterobacteriaceae as well as for coliphage plaque assays. TSB is commercially available otherwise prepare as follows: add 10 g of Tryptone; 5 g of Yeast Extract and 10 g of NaCl to 1 L of distilled water. For the TSA plates, prepare TSA (16 g TSB; 1.2-1.5 % agar; 800 mL of distilled water), autoclave, pour in petri dishes and let it solidify.
2. MTA is generally prepared with 0.6 % of agar however agar percentages between 0.4 and 0.7 % can be used. Alternatively, MTA can be prepared with agarose instead of agar; MTA 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.
3. Optional: 2 % of gelatin (w/v) can be added to SM buffer. Gelatin is known to preserve phages and thus can be used in the later steps of phage purification.
4. A loopful of freshly grown host bacterium can be picked from agar plates and suspended in saline solution (0.9 % NaCl) and used instead of overnight grown bacterial suspension.
5. The culture media and the temperature depend on the bacteria species used.
6. Several different phage enrichment samples, from different sources and origins, can be spotted on one bacterial lawn.
7. The paper strip has the same functionality for streaking phages as the inoculating loops have for streaking colonies.
8. 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.
9. The MTA\_TSB layer can also be collected.
10. 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.
11. Nucleic acids have absorbance maxima at 260 nm. A 260/280 ratio of ~1.8 is generally accepted as “pure” for DNA. 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 organic compounds contamination.
12. Reduced voltage increases gel resolution.

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## SUPPORTING INFORMATION

### Annex I. PHAGE PURIFICATION

#### 1. INTRODUCTION

Phages can be purified from crude bacterial lysates by using several methods<sup>1</sup>. The addition of 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.

#### 2. MATERIALS

##### 2.1. PEG purification

- SM buffer
- Chloroform
- NaCl, 99.5 %
- PEG 8,000 (MW 5,000 - 7,000 g/mol)
- Centrifuge tubes
- PES Filters (0.2 µm)
- 50 mL Falcon tubes

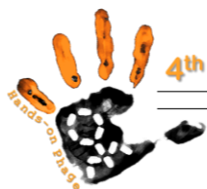
##### 2.2. CsCl purification

- Cesium chloride: CsCl, 99.9 %
- SM buffer
- Chloroform
- Ultracentrifuge tubes (see **note 1**)
- Syringes and 18-22 gauge hypodermic needles
- Dialysis membranes: MWCO 12-14,000

#### 3. METHODS

##### 3.1. PEG purification

1. After transferring the SM buffer with the eluted phages to 50 mL Falcon tubes (step 3.4.2), dissolve solid NaCl into the phage suspension to the concentration of 0.5 M and let it cool at 4 °C for 1h;
2. Remove denser bacterial debris by centrifugation of 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 % at 4 °C, by brief stirring, and let it stand at 4 °C for at least overnight in order to precipitate phage particles;
4. Sediment the precipitated phage at 10,000 × g for 15 min at 4 °C and carefully discard the supernatant;



5. Turn the centrifuge bottles over and let the remaining fluid drain away from the pellet for 5 min;
6. Gently suspend the pellet in phage SM buffer (1-3 mL per 100 mL of supernatant). Since phage particles may be damaged by vortexing or vigorous pipetting, it is recommended to leave it overnight at 4 °C in order to soften, 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 it is not needed to go any further in the purification, 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 (0.2 µm) and transfer to sterile tubes;
10. Store at 4 °C until further use.

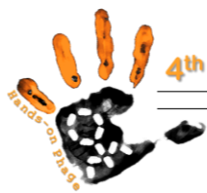
### 3.2. CsCl purification

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

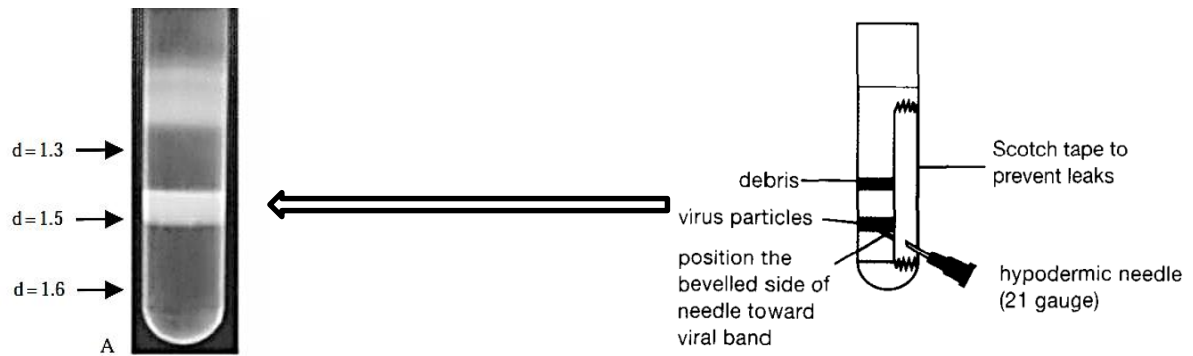
**Table S1 - CsCl solutions currently used for phage purification**

| <i>d</i> (g/mL) | <i>CsCl</i> (g/mL) | <i>n</i> (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 0.50 g of solid CsCl per mL of phage sample and agitate until the salt is dissolved;
3. Prepare step gradients: Layer on the centrifuge tube, first the solution with the lowest density then the next heavier using a long canular needle and so on until the most heavier solution; then layer the phage sample on top;
4. Once the gradient is ready, add the phage suspensions to the top of the gradient;
5. Centrifuge at 22,000–25,000 rpm (relative centrifugal field of 100,000–120,000 × g) for 2–3 h at 4 °C;
6. Collect the phage band by puncturing the side of the tube with a hypodermic needle (**Figure S1**);
7. Remove the CsCl from the phage suspensions by dialysis at 4 °C, two or three times for 30 min against 500 volumes of SM buffer or overnight against 2,000 volumes of SM buffer;
8. Store the dialyzed phage suspension at 4 °C with a few drops of chloroform (if the phage tolerates chloroform);
9. If required, to separate phages from contaminated RNA and DNA, a second CsCl centrifugation can be performed;
10. For this, place the phage suspension from 6 in an ultracentrifuge tube and fill with CsCl solution (density equal to 1.5 g/mL in SM) for 24h at 4 °C;



11. Collect the band as described previously and store the sample at 4°C;
12. Dialyse the sample to remove the CsCl.



**Figure S1** – Purification of bacteriophages by CsCl centrifugation. (A) CsCl step gradient: bacteriophage (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 bacteriophages by side puncture (a tape was attached outside the tube, level with the phage band to prevent leakage around the needle) (adapted from Sambrook and Russell<sup>1</sup>).

#### 4. REFERENCES

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## Annex II. PHENOL/CHLOROFORM DNA EXTRACTION

### 1. INTRODUCTION

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<sup>1,2</sup>.

### 2. MATERIALS

- Phage sample (see note 1)
- 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)
- Proteinase K
- 0.5 M EDTA, pH 8.0
- 10 % SDS (w/v)
- Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v)
- Chloroform
- Isopropanol
- 70 % ethanol
- 3M sodium acetate (pH 4.6)
- Ultrapure water
- Waterbath or heating plate with controlled temperature

### 3. METHODS

1. Add 400 µL of L1 Buffer to each 250 mL of phage sample and incubate for 2 h at 37 °C;
2. Incubate the sample for 15 min at 70 °C;
3. Add 1 % SDS, 20 mM EDTA and 50 µg 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 3000 xg, 15 min, room temperature;
6. Carefully remove the upper phase. (see note 2);
7. Repeat steps 5 and 6;
8. Add 1 vol of chloroform and centrifuge at 1600 xg, 15 min, room temperature;
9. Carefully remove 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 xg, 10 min, 4 °C;
13. Wash the pellet with 70 % 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.

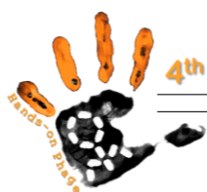


#### 4. NOTES

1. It is recommended to work with high concentrations ( $\geq 10^{10}$  PFU/mL) of polyethylene glycol (PEG) or ideally cesium chloride purified phages 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 remove 80-90 % of the aqueous phase in the initial extraction.

#### 5. REFERENCES

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## PRACTICAL SESSION P2

### Bacteriophage genome annotation

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#### 1. INTRODUCTION

We live in a sequencing revolution era where over 2,000 completely sequenced bacteriophage (phage) genomes has already been accomplished. 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<sup>1</sup>. Despite a steady 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: phage genome sequencing, annotation and describing functions to phage genes, prophage identification in bacterial sequences, elucidating the events in various stages of phage life cycle using genomic, transcriptomic and proteomic approaches, defining the mechanisms of host takeover including specific bacterial-phage protein interactions, identifying virulence and other adaptive features encoded by phages<sup>2</sup>.

#### 2. METHODS

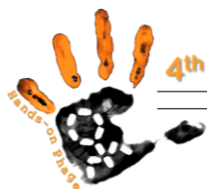
Several user-friendly software are currently available and were designed and developed to fulfill the needs of biologists to analyze large datasets. Most of the software programs use sophisticated computational methods and Table 1 provides a brief catalogue of some analysis tools, many of which will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

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

| Name               | Summary  | Application   | License & cost  |
|--------------------|--|---|-----------------|
| <b>ARAGORN</b>     | Search for tRNA and show their positioning                   | Search for transfer RNA (tRNA)  | Free            |
| <b>ARNold</b>      | Search for transcription terminators                         | Finds rho-independent terminators in nucleic acid sequences   | Free            |
| <b>Bionumerics</b> | Search, organize and analyze genomic and protein information | Assembly, annotation, comprehensive sequence analysis, annotation, etc  | License – 7000€ |
| <b>BLAST</b>       | Search for homologs  | BLAST finds regions of similarity between biological sequences. The program compares nucleotide or protein sequences to | Free            |

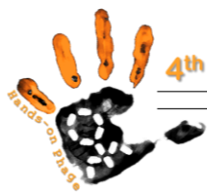


| Name                         | Summary   | Application  | License & cost  |
|------------------------------|---|--|---|
|                              |   | sequence databases and calculates the statistical significance.  |   |
| <b>Compute pI/Mw</b>         | Calculates protein pi and Mw                                      | Determines each protein isoelectric point and molecular weight   | Free  |
| <b>Geneious</b>              | Search, organize and analyze genomic and protein information      | Assembly, alignment, annotation and prediction, etc.   | 15-day trial version. Academic and non-academic license |
| <b>CLC Genomic Workbench</b> | Search, organize and analyze genomic and protein information      | Assembly, alignment, annotation and prediction, etc.   | 12-day trial version. Academic and non-academic license |
| <b>HHpred</b>                | Homology detection and structure prediction by HMM-HMM comparison | Protein function and protein structure prediction server that is based on HHsearch and HHblits   | Free  |
| <b>InterProScan</b>          | Protein Domain Prediction   | Allows you to scan your sequence for matches against the InterPro protein signature databases, using InterProScan tool.  | Free  |
| <b>MEME/MAST</b>             | Predicts putative transcription promoters                         | Align ORF upstream sequences and discovers conserved motifs within these sequences. Furthermore allows a graphical representation of putative promoter sequences   | Free  |
| <b>Mfold</b>                 | Predicts the folding of terminator sequences                      | Allows the observation of the folding structure of a putative terminator   | Free  |
| <b>myRAST</b>                | <i>In silico</i> annotation of genomes                            | Automatic annotation of a genome   | Free  |
| <b>Pfam</b>                  | Search for protein families                                       | Search homologies of query protein with Pfam database that has a large collection of protein families  | Free  |
| <b>PHIRE</b>                 | Predicts putative transcription promoters                         | Performs an algorithmic string-based search on phage genome sequences, discovering and extracting blocks displaying sequence similarity, corresponding to conserved regulatory elements contained within these genomes | Free  |
| <b>Phobius</b>               | Search for transmembrane domains                                  | Determines the number of transmembrane domains that each protein has   | Free  |



| Name               | Summary   | Application  | License & cost |
|--------------------|---|--|----------------|
| <b>TMHMM</b>       | Search for transmembrane domains  | Determines the number of transmembrane domains that each protein has | Free           |
| <b>TransTermHP</b> | Search for transcription terminators  | Finds rho-independent terminators in nucleic acid sequences          | Free           |
| <b>tRNAscan-SE</b> | Search for tRNA and show their positioning  | Search for transfer RNA (tRNA)                                       | Free           |
| <b>SignalP</b>     | Predicts the presence and location of signal peptide cleavage in amino acid sequences | Search of possible signal peptide cleavage sites                     | Free           |
| <b>CoreGenes</b>   | Analysis of genes or proteins in common from sets of up to five genomes               | Finds proteome conservation  | Free           |
| <b>HHMER</b>       | Search sequence databases for sequence homologs, and sequence alignments              | Search of homologs   | Free           |
| <b>OrthoVenn</b>   | Genome wide comparison and annotation of orthologous clusters across multiple species | Search of orthologs  | Free           |

Although the software programs described in this table are extremely useful, the generated data should always be checked manually. Briefly, in this practical session we will start with an assembled genome sequence and go through an automatic annotation which will be improved manually for alternative start codons, non-annotated CDSs, possible new or alternative and more reliable protein functions<sup>3</sup>. Regulatory elements will also be scanned as well some basic protein parameters. Finishing the genome annotation, some comparative genomics can also be carried in order to identify and understand related phage genomes/proteomes already deposited in the common databases.



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## PRACTICAL SESSION P3

### Phage display technology

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#### 1. INTRODUCTION

Phage Display is a powerful, high-throughput technology used to identify interacting molecules and ligands for a given target. Described in 1985<sup>1</sup>, Phage Display allowed Professor George P. Smith to be awarded in 2018 with the Nobel Prize of Chemistry by the insertion of a specific peptide/protein sequence into the coat protein gene of a bacteriophage (phage), with consequent expression on the outside of the phage.

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<sup>2-5</sup>. 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 bacteriophages 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 bacteriophage 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 bacterial 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)<sup>6-11</sup>.

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<sup>12,13,14,15</sup> 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<sup>16</sup>. 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 (**Figure 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<sup>9,16</sup>.

## 2. MATERIALS

### 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% trypsin: 0.02% EDTA)
- 1X PBS (Phosphate Buffered Saline) pH7.4
  - **10X PBS:**  
For 250 mL weight 20 g of NaCl, 0.5 g of KCl, 3.6 g of Na<sub>2</sub>HPO<sub>4</sub>, 0.6 g of KH<sub>2</sub>PO<sub>4</sub> and add distilled water up to 200 mL.  
Adjust the pH to 7.4 with HCl.  
Adjust the volume to 250 mL with distilled water.  
Sterile by autoclaving at 121 °C and 1 bar for 20 minutes.  
Store at room temperature.  
Dilute 10X to use as 1X and store at room temperature.
- Sterile plastic Pasteur pipettes
- Disposable and sterile 5 mL and 10 mL pipettes
- Sterile 1.5 mL Eppendorf tubes
- Sterile 15 mL Falcon tubes
- Neubauer chamber
- Coverslips
- Micropipettes and tips
- Microscope
- Centrifuge for 15 mL Falcon tubes

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

- Dulbecco's Modified Eagle Medium (DMEM)
- Bovine Serum Albumin (BSA)
- **DMEM + 1 % BSA and DMEM + 3 % BSA:**  
For 50 mL add 50 mL of DMEM + 0.5 g and 1.5 g of BSA, respectively.  
Dissolve by heating in a 37 °C water bath.  
Sterilize by filtering with a 0.22 µm filter.  
Store at 4 °C.
- **Organic phase 9:1 (v/v) cyclohexane:dibutyl phthalate:**  
For 10 mL add 9 mL dibutylphthalate and 1 mL cyclohexane



- **1M Tris-HCl pH9.1:**  
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.  
Sterilize by autoclaving at 121 °C and 1 bar for 20 minutes.  
Store at room temperature.
- Sterile 50 mL Falcon tubes
- Sterile 15 mL Falcon tubes
- Sterile 0.22 µm filters
- PhD-12 library (New England Biolabs)

### 2.3. Phage Titer Assay

- **TSB (Tryptic Soy Broth) medium:**  
For 400 mL weight 12 g of commercially available TSB and add distilled water up to 400 mL.  
Sterilize by autoclaving at 121 °C and 1 bar for 20 minutes.  
Store at room temperature.
- **0.7 % Molten Top Agar (MTA) (see note 1):**  
For 400 mL weight 12 g of TSB and 2.8 g of agar and add distilled water up to 400 mL.  
Sterilize by autoclaving at 121 °C and 1 bar for 20 minutes.  
Store at room temperature (see note 2).
- **TSB+X-Gal+IPTG plates:**  
For 400 mL weight 12 g of TSB and 8 g of agar and add distilled water up to 400 mL.  
Sterilize by autoclaving at 121 °C and 1 bar for 20 minutes.  
Let the temperature drop to 50 °C and add 800 µL of 20 mg/mL X-Gal and 80 µL of 1M IPTG  
Spread 25-30 mL of medium per plate. Let it dry inside a laminar flow chamber.  
Store at 4 °C.
- *Escherichia coli* K12 strain ER2738
- Sterile 15 mL Falcon tubes
- Sterile 1.5 mL Eppendorf tubes

### 2.4. ssDNA Isolation

- TSB medium
- ER2738 *E. coli* strain
- **20% 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 with a 0.22 µm filter.  
Store at room temperature.
- **Iodide Buffer:**  
For 10 mL add 8 mL of 5M sodium iodide (NaI), 100 µL of 1M Tris-HCl pH8, 20 µL of 0.5M EDTA pH8 and add distilled water up to 10 mL.  
Filter sterilize with a 0.22 µm filter.  
Store at 4 °C protected from light.
- 100% ethanol (cold)
- 70% ethanol (cold)



- 1X TE (Tris-EDTA buffer) pH8
- **10X TE:**  
For 50 mL add 500 µL 1M Tris-HCl pH8, 100 µL 0.5M EDTA pH8 and add distilled water up to 50 mL.  
Sterile by autoclaving at 121 °C and 1 bar for 20 minutes.  
Store at room temperature.  
Dilute 10X to use as 1X and store at room temperature.
- Sterile 15 mL Falcon tubes
- Sterile 1.5 mL Eppendorf tubes
- Sterile wooden sticks
- Sterile 0.22 µm filters

### 3. METHODS

#### 3.1. Cell detachment

1. Grow the control and target cell lines in T-25 flasks in 3 mL of the appropriate medium in a CO<sub>2</sub> 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 1X pH7.4;
3. Detach the cells using 500 µL of trypsin-EDTA solution and incubate in the CO<sub>2</sub> incubator for 5 minutes or until all the cells are detached;
4. Add 500 µL of DMEM + 1% BSA to remove all the cells from the flask surface and transfer the suspension to a 15 mL tube;
5. Put 10 µL in a Neubauer chamber to determine the cell number as follows:  
$$N \text{ (cell number)} = \text{number of cells counted} / \text{number of squares} \times 10000 \times \text{volume}$$
  
The cell concentration must be adjusted to around  $1 \times 10^6$  cells/mL.
6. Centrifuge the cell suspension for 5 minutes at 1400 rpm at room temperature;
7. Remove the supernatant and add the same volume of DMEM + 1% BSA;  
Repeat steps 6 and 7 twice.
8. Remove all the supernatant and resuspend the pellet with 200 µL of pre-chilled DMEM + 3% BSA and transfer to a new 1.5 mL tube. Keep the 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 hours, **Figure 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 falcon tube], containing the organic phase 9:1 (v/v) cyclohexane:dibutyl phthalate submerging the interior tube;
3. Add 500 µL of pre-chilled DMEM + 1% 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 minutes at 10000 *g* and 4°C (**Figure 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, **Figure 1C**.

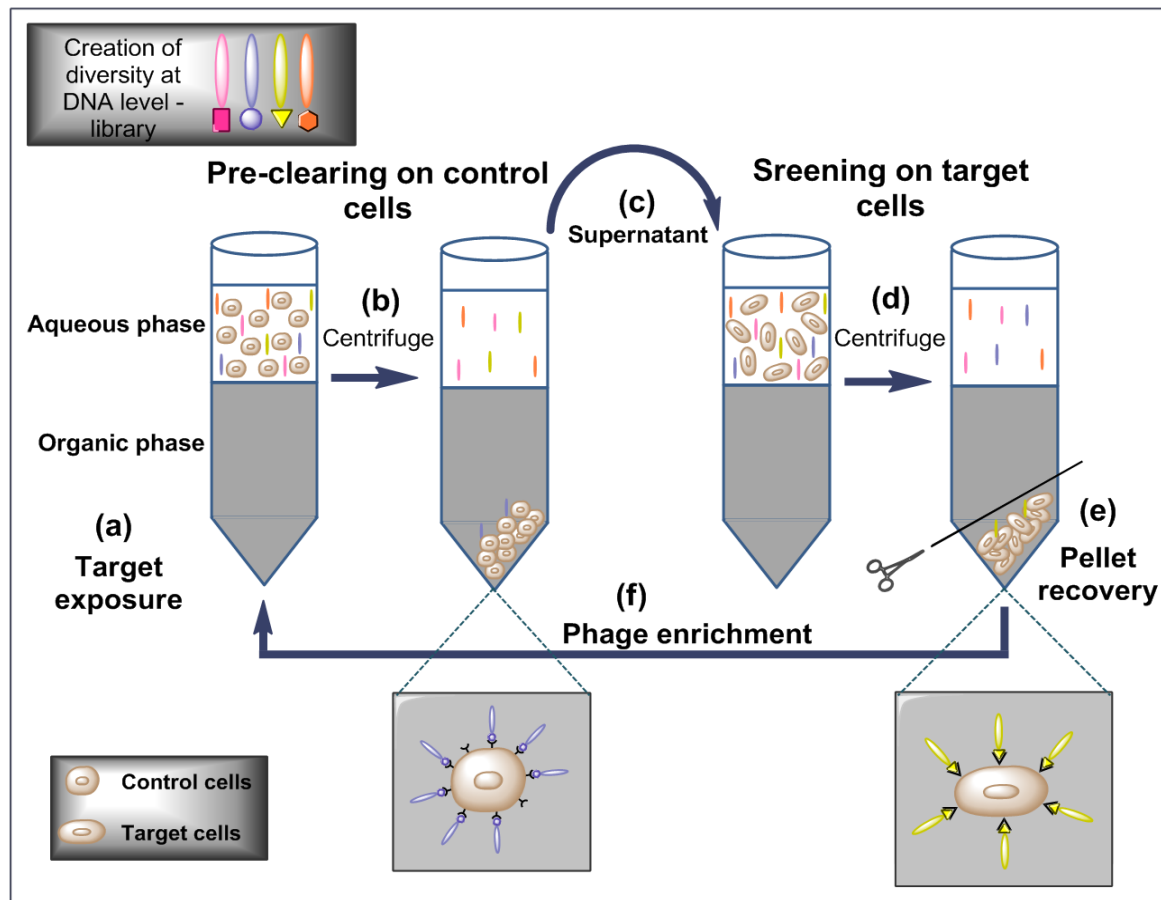


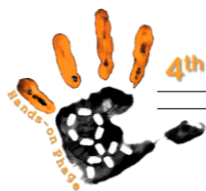
Figure 1. BRASIL methodology.

### 3.2.2. Screening step (target cells):

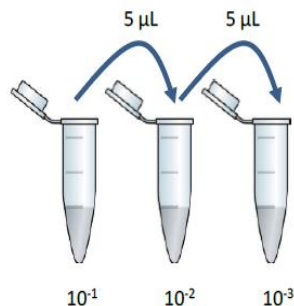
1. Add the unbound phage pool obtained from the supernatant (droplet) of the pre-clearing step to 200  $\mu\text{L}$  of the target cells at  $1 \times 10^6$  cells/mL, and incubate on ice for 4 hours, **Figure 1C**;
2. Prepare the "BRASIL tube" as described for the pre-clearing step;
3. Add 500  $\mu\text{L}$  of pre-chilled DMEM + 1% 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 minutes at 10000  $g$  and 4  $^{\circ}\text{C}$ . Remove the droplet and the organic phase and resuspend the pellet with 50  $\mu\text{L}$  of 1M Tris-HCl buffer pH9.1. Put in a new 1.5 mL tube and store at 4  $^{\circ}\text{C}$  for phage titer, **Figure 1E**.

### 3.3. Phage titer assay

1. Inoculate 5 mL of TSB with ER2738 from a plate and incubate at 37  $^{\circ}\text{C}$  with shaking for 5 h until mid-log phase, OD600  $\sim$  0.5.



2. Prepare successive  $10^{-1}$ ,  $10^{-2}$  and  $10^{-3}$ -fold serial dilutions (1:10) of the eluted phage in TSB medium (add 50  $\mu$ L of the eluted phage and 450  $\mu$ L of TSB in 1.5 mL Eppendorf tubes) as follows:



3. In new 1.5 mL tubes add 10  $\mu$ L of the dilutions to 200  $\mu$ L of *E. coli* ER2738, mix and incubate 1-5 minutes;
4. In a 15 mL Falcon tube add 3 mL of 0.7% MTA and all the previous suspension (10  $\mu$ L of the dilutions + 200  $\mu$ L 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 bacteriophage plaques in the dilution which resulted in 20-200 plaques and determine the titer of triplicate preparations according to the equation:

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

**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, **Figure 1F**.

### 3.4. ssDNA isolation

1. Dilute an overnight culture of ER2738 1:100 in TSB and dispense 1 mL into 15 mL Falcon tubes, one for each clone to be characterized;
2. Use a sterile wooden stick to stab a blue plaque from a titrating plate (see **note 3**) and transfer to a tube containing the diluted culture. Incubate the tubes at 37 °C with shaking for 4.5 hours;
3. Transfer the cultures to 1.5 mL Eppendorf tubes and centrifuge at 14.000 rpm for 30 seconds and transfer 500  $\mu$ L of the phage-containing supernatant to a new 1.5 mL Eppendorf tube;
4. Add 200  $\mu$ L of 20% PEG/2.5 M NaCl. Invert several times to mix, and let stand for 10-20 minutes at room temperature;



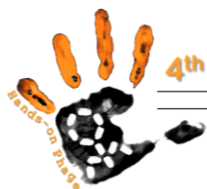
5. Centrifuge at 14.000 rpm for 10 minutes at 4 °C and discard the supernatant, re-spin briefly and carefully pipet away and discard any remaining supernatant;
6. Suspend the pellet thoroughly in 100 µL of iodide buffer by vigorously tapping the tube. Add 250 µL of 100% ethanol and incubate 10-20 minutes at room temperature (see **note 4**);
7. Spin at 14.000 rpm for 10 minutes at 4°C, and discard the supernatant. Wash the pellet with 0.5 mL of chilled 70% ethanol, re-spin, discard the supernatant, and briefly dry the pellet;
8. Suspend the pellet in 30 µL of TE buffer (see **note 5**) and quantify 1-2 µL in a NanoDrop spectrophotometer.

#### 4. NOTES

1. MTA is generally prepared with 0.6% agar but agar percentages between 0.4 and 0.7 can be used. Alternatively, MTA can be prepared with agarose instead of agar.
2. 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.
3. Plates should be <1-3 days old, stored at 4 °C and have <100 plaques. Pick well-separated plaques. This will ensure that each plaque contains a single DNA sequence.
4. Short incubation at room temperature will preferentially precipitate single-stranded phage DNA, leaving most phage protein in solution.
5. The template can be suspended in H<sub>2</sub>O 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.

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## PRACTICAL SESSION P4

### Monitoring bacteriophage/host interaction

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#### 1. INTRODUCTION

Phages are useful in a wide range of applications, from agriculture and foodstuff industries to health facilities to combat bacterial infections. Despite the success on several phage applications, there were also some unsuccessful results. Consequently, the study of phage-host interactions is of utmost importance to provide knowledge to the field and thus select the best phages for further applications. Recently, phage-host interactions were analyzed through gene expression studies and also by a novel flow cytometry approach<sup>1</sup>.

Quantitative PCR (qPCR) is a technique widely used to study gene expression. In the phage-host interaction scenario, RNA is extracted on several time points and converted into complementary DNA (cDNA). Generally, gene expression is assessed by relative expression of a target gene in comparison with a reference gene (e.g. 16S rRNA).

Flow cytometry is a technique that allows a quick, accurate and highly reproducible analysis of individual cells within a population<sup>2</sup>. In comparison, with culture-dependent methods, flow cytometry allows to detect viable but non-culturable bacterial cells, and also to assess parameters such as morphological/physiological diversity of populations<sup>3</sup>.

#### 2. MATERIALS

Prepare all solutions using distilled water. The media used in the procedures described herein is Tryptic Soy Broth (TSB) 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 materials and store them at room temperature (unless indicated otherwise).

##### 2.1. Bacteriophage Infection of Planktonic Cultures

- Purified bacteriophage suspension (see **Note 1**);
- TSB broth prepared according to the manufacturer's instructions (see **Note 2**);
- Bacterial culture: Place one colony of the bacterial host into a glass flask with 25 mL of TSB and incubate at appropriate temperature for 16 hours at 120–150 rpm (see **Note 3**);
- Exponential phase culture: Dilute the overnight grown culture 1:100 (vol/vol) with TSB to a final volume of 50 mL and incubate at appropriate temperature with agitation until reaches an optical density at 600 nm ( $OD_{600nm}$ ) of approximately 0.6 ( $\approx 6 \times 10^8$  CFU.mL<sup>-1</sup>) (see **Note 4**);
- Saline Magnesium buffer (SM buffer): 100 mM NaCl, 8 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, and 50 mM Tris-HCl, pH 7.5;
- Sterile 100 mL glass flasks;
- Sterile 50 mL tubes;
- Syringes;
- Syringe filters (0.22 µm).



## 2.2. RNA extraction

- Bacterial planktonic cultures;
- Purified bacteriophage suspension;
- RNase Xterminator Spray (Grisp) (see **Note 5**);
- RNaprotect Bacteria Reagent (Qiagen) (see **Note 6**);
- EXTRACTME RNA BACTERIA & YEAST KIT (DNA-Gdansk) (see **Note 7**);
- 150-212  $\mu\text{m}$  Glass Beads (Sigma);
- 2.0 mL Screw Cap Tube (BIOplastics)
- Screw Caps (BIOplastics)
- FastPrep Cell Disruptor (see **Note 8**);
- Centrifuge (refrigerated);
- Ethanol 70% (vol/vol);
- Nuclease-free 1.5 mL microcentrifuge tubes;
- Nuclease-free water;
- DNase I, RNase-free (ThermoScientific).

## 2.3. cDNA Synthesis

- Xpert cDNA Synthesis Mastermix (Grisp) (see **Note 9**);
- Template RNA (see **Note 10**);
- Nuclease-free water.

## 2.4. Gene expression quantification

- Xpert Fast SYBR (Grisp);
- 100 $\times$  ROX (50 $\mu\text{M}$ ) reference dye (see **Note 11**);
- Primers (5 pmol/ $\mu\text{L}$  each);
- Nuclease-free water;
- Template cDNA;
- Quantitative Thermal cycler.

## 2.5. Flow Cytometry Analysis

- Purified bacteriophage suspension;
- Bacterial planktonic cultures;
- Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , 1.8 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4;
- Vortex;
- SYBR Green (SYBR): 1:40,000 of SYBR Green I (Invitrogen);
- Propidium Iodide (PI): 20  $\mu\text{g}/\text{mL}$  propidium iodide (Sigma- Aldrich) (see **Note 12**);
- Sterile 3 mL polypropylene tubes: 75  $\times$  10 mm;
- Flow cytometer.



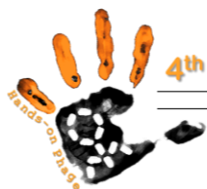
### 3. METHODS

#### 3.1. Bacteriophage Infection of Planktonic Cultures

1. Dilute 1:100 (vol/vol) a 16 h bacterial inoculum with TSB to a final volume of 50 mL, and incubate in a 100 mL flask at appropriate temperature with agitation (120–150 rpm) until an OD<sub>600nm</sub> of approximately 0.4–0.5.
2. Add the bacteriophage suspension to the bacterial culture in order to obtain the multiplicity of infection (MOI) required (see **Note 13**). In control experiments, use SM Buffer instead of bacteriophage suspension.
3. Incubate the suspension at appropriate temperature with agitation (120–150 rpm) and take samples at different time points for further analysis.

#### 3.2. RNA extraction

1. Harvest bacteria by centrifugation (not more than  $1 \times 10^9$  cells) at a maximum speed for 5 minutes at 4 °C (see **Note 14**);
2. Discard supernatant and suspend the pellet in 600 µL RYBL buffer (see **Note 15**);
3. Transfer the suspension into 2 mL safeLock tubes with 0.5 g of glass beads. Label the tubes on the lateral (FastPrep cell disruptor erases the wink from the top of the tubes). Ensure tubes are well closed to avoid spills;
4. Place the tubes into FastPrep Cell disruptor and set the equipment for 35 seconds at 6.5 m/s. Place the samples immediately on ice and let them cool for 5 minutes. Repeat this cycle 4 times;
5. Centrifuge the tubes at max speed for 3 minutes at 4 °C at RT (see **Note 16**);
6. Transfer the suspension (avoiding the aspiration of glass beads) into a RNA Homogenization Column H and centrifuge for 2 minutes at 13,000 ×g. Keep the flow through and add equal volume of 70% ethanol directly into the collection tube (see **Note 17**). Mix by pipetting up and down;
7. Transfer 700 µL of the mixture (including any precipitate) into an RNA Purification Column B placed in a collection tube. Centrifuge for 1 min at 13,000 ×g. Discard the flow-through and place the column in to a new collection tube. The maximum capacity of this column system is 700 µL, hence, if you have more than 700 µL you have to perform this step again until all your RNA suspension has passed through the column;
8. Add 650 µL of RYBW1 Buffer to the column. Centrifuge for 1 min at 13,000 ×g. Discard the flow-through and reuse the collection tube;
9. Add 650 µL of RYBW2 Buffer to the column. Centrifuge for 1 min at 13,000 ×g. Discard the flow-through and reuse the collection tube;
10. Add 500 µL of RYBW2 Buffer to the column. Centrifuge for 1 min at 13,000 ×g. Discard the flow-through and reuse the collection tube;
11. Centrifuge for 2 more minutes at 13,000 ×g to remove any trace of ethanol, which is known to influence downstream applications;
12. Discard the flow-through and the collection tube. Carefully transfer the column to a 1.5 mL DNase/RNase free tube;
13. Add 50 µL of nuclease-free water precisely onto the center of the column membrane. Incubate at RT for 3 min and centrifuge at 13,000 ×g for 2 min (see **Note 18**);
14. To 50 µL RNA add 5 µL of DNase I buffer (10×) and 2 µL of DNase. Mix very well by pipetting up and down (see **Note 19**);
15. Incubate for 30 minutes at 37 °C;
16. Add 5 µL of EDTA to the samples and mix well by pipetting up and down;



17. Incubate for 10 minutes at 65 °C (see **Note 20**);
18. Immediately place RNA on ice. Alternatively, RNA can be safely stored at this point. For short storage purposes place RNA at -20 °C, or at -80 °C for longer storage purposes.

### 3.3 cDNA Synthesis

1. Measure the RNA concentration on a Nanodrop;
2. Mix the following components in a RNase-free microtube;

| Component           | Volume              |
|---------------------|---------------------|
| Template RNA        | 250 ng of total RNA |
| MasterMix (2×)      | 10 µL               |
| Nuclease-Free water | Up to 19 µL         |

3. Use a thermocycler to heat samples at 65 °C for 5 min;
4. Place on ice for 2 min;
5. Add 1 µL of Xpert RTase (200 U/µL) to the mixture (see **Note 21**);
6. Mix thoroughly and then centrifuge briefly;
7. Incubate at 25 °C for 10min;
8. Using a thermocycler or thermoblock, heat the microtube for 15 min at 50 °C;
9. Inactivate Enzyme by heating for 5 min at 85 °C;
10. Either use cDNA immediately as template in qPCR/PCR or store at -20 °C.

### 3.4. Gene expression quantification

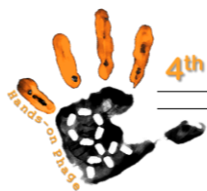
1. Dilute cDNA (1:100) to a Work Solution (see **Note 22**);
2. For each PCR reaction mix (see **Note 23**):

| Component   | Volume (µL) | Final concentration |
|---|-------------|---------------------|
| Xpert Fast SYBR 2× Mastermix (uni) with ROX (see <b>Note 24</b> ) | 10 µL       | 1×                  |
| Forward Primer (5 pmol/µL) (see <b>Note 25</b> )                  | <2 µL       | 50-400 nM           |
| Reverse Primer (5 pmol/µL) (see <b>Note 25</b> )                  | <2 µL       | 50-400 nM           |
| Template DNA (see <b>Note 26</b> )                                | <6 µL       | <100 ng             |
| Nuclease-Free water   | Up to 20 µL | -                   |

3. Set-up qPCR cycling with the following instructions:

| Number of cycles                    | Temperature                            | Time      |
|-------------------------------------|--|-----------|
| 1×                                  | 95 °C                                  | 2 min     |
| 40×                                 | 95 °C                                  | 5 sec     |
|                                     | 60-65 °C                               | 20-30 sec |
| Melt Analysis (see <b>Note 27</b> ) | According to manufacturer's guidelines |           |

4. Quantify mRNA transcripts, for each gene under study, using the Pfaffl method<sup>4</sup> (see **Note 28**).



### 3.5. Flow Cytometry Analysis

1. Open a new protocol in the flow cytometer software;
2. Set the plots listed below, on logarithmic scale, for bacteria visualization:
  - (a) Forward Scatter (FSC) vs. Side Scatter (SSC)—relative size vs. granularity;
  - (b) SYBR vs. PI.
3. Set the volume of sample to be analyzed to 1/4 of the total sample volume (e.g. 50  $\mu\text{L}$  of a 200  $\mu\text{L}$  sample);
4. Set the flow rate to low (e.g. 10  $\mu\text{L}/\text{min}$  as used in DNA analysis);
5. Acquire 200  $\mu\text{L}$  of PBS suspension to define the background in the FSC vs. SSC dot plot;
6. Add 20  $\mu\text{L}$  of planktonic suspension (1:10 diluted), into a PP tube with 180  $\mu\text{L}$  of PBS and acquire on the flow cytometer (see **Note 29**);
7. Gate all dot plots in the bacterial population; Adjust adequately the voltage and the gains so the unstained bacteria are on the Q1 region;
8. Add 20  $\mu\text{L}$  of planktonic suspension (1:10 diluted), into a PP tube with 180  $\mu\text{L}$  of a solution containing 1:40,000 of SYBR (see **Note 30**);
9. In a new PP tube add 20  $\mu\text{L}$  of planktonic suspension (1:10 diluted) and 180  $\mu\text{L}$  of a solution containing 20  $\mu\text{g}/\text{mL}$  of PI (see **Note 30**);
10. Vortex and incubate all samples for 5–20 min at room temperature and protected from the light (see **Note 31**);
11. Acquire single-stained samples and set the compensations if necessary;
12. Add 20  $\mu\text{L}$  of each bacterial suspension (1:10 diluted) into a PP tube with 180  $\mu\text{L}$  of a solution containing 1:40,000 of SYBR and 20  $\mu\text{g}/\text{mL}$  of PI;
13. Vortex and incubate for 5–20 min all samples at room temperature and protected from the light;
14. Acquire double-stained samples. The compensation values optimized with unstained and single-stained cells should be adequate, but fine-tune alterations may be necessary;
15. Analyze the obtained data regarding:
  - (a) SYBR Median fluorescence intensity and if an increase in intensity is observed this is indicative of increased metabolic state;
  - (b) Cell counts/ $\mu\text{L}$  (see **Note 32**);
  - (c) Number of intact, compromised, and dead cells.

### 4. NOTES

1. To purify the bacteriophages, use the method described by Sambrook and Russell<sup>5</sup>.
2. 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 sodium chloride (NaCl); 2.5 g of dipotassium phosphate ( $\text{K}_2\text{HPO}_4$ ); and 2.5 g of Dextrose.
3. The procedure is described for fast growing bacteria. However, if experiments are performed with slow growing bacteria, the incubation time needs to be adjusted.
4. OD vs CFUs should be previously studied. Values are different between bacteria.
5. RNase Xterminator Spray is a secondary alcohol based solution containing anionic surfactants for the inactivation and removal of RNases (and other enzymes) as well as DNA from laboratory surfaces. The

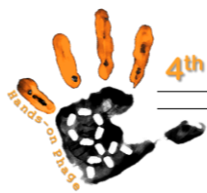


solution can be used to decontaminate bench tops, labware, pipettors, and other equipment. It is effective on metal, glass and plastic surfaces.

6. Traditional methods for cell harvesting and RNA isolation can lead to vast changes in bacterial expression profiles. Enzymatic degradation of RNA leads to reduction or loss of many transcripts because bacterial mRNA molecules have very short half-lives of only a few minutes. In addition, genes can be induced during handling and processing of samples, leading to higher expression levels of certain transcripts. Use of RNeasy Protect Bacteria Reagent overcomes these problems by providing immediate stabilization of RNA prior to RNA isolation procedures.
7. Other kits can be used to extract RNA, optimize them to your microorganism.
8. In the lack of this equipment, a vortex can be used. Time of vortex and number of vortex cycles should be optimized to maximize nucleic acid extraction but also to minimize nucleic acid damage.
9. The Xpert cDNA Mastermix (2X) is an optimized reaction mix containing a balanced concentration of oligo(dT) and random hexamer primers, dNTPs, and RNase inhibitor. Note that, since the mastermix already contains primers, it cannot be used with gene-specific primers. First strand cDNA can be directly used as template in PCR.
10. It can be used 1 ng - 2 µg of total RNA.
11. Depending on your equipment, prior to use for the first time, add 2 µl ("LowROX") or 20 µl ("HighROX") of the 100× ROX reference dye to the mastermix and vortex briefly. If your instrument is "No ROX", then you should use the mastermix as it is, thus without addition of ROX.
12. Bacterial cells stained with dual-color LIVE/DEAD® can be run in a flow cytometer for analysis of their viability. This dual-stain system reports on cell viability via membrane integrity, and can be used to measure the viability of bacteria growing in biofilm communities. The LIVE/DEAD® kit makes use of the different permeability of the green SYTO9 and red propidium iodide (PI) dyes<sup>6</sup>. While SYTO9 penetrates the membrane of all cells and binds their DNA, PI can only penetrate damaged membranes<sup>6</sup>. Since PI exhibits a stronger affinity for nucleic acids, SYTO9 is displaced by PI and, consequently, live cells will be stained with green and dead cells with a red fluorescence<sup>6,7</sup>. In alternative to SYTO, SYBR green can also be used as a component of the LIVE/DEAD staining to assess the cell viability by flow cytometry<sup>6,8</sup>. Furthermore, it was reported that this fluorochrome can be used to assess the physiological state of bacterial cells<sup>9</sup>.
13. To guarantee uniform results, it is recommended to use a MOI enough to obtain a synchronized infection<sup>10</sup>.
14. Keep in mind that sample collection has to be fast, as the changes made during sample collection can impact gene expression levels, being low expressed genes particularly vulnerable. As alternative, you can use reagents that "freeze" cells (RNA protect Bacteria reagent, RNA later or similar reagents), thus maintaining gene expression levels stable during the whole procedure.
15. β-mercaptoethanol is a RNase inhibitor that can be added to the RYBL buffer. To do so, add 10 µL of β-mercaptoethanol per 1 mL of RYBL buffer. This solution is stable for 1 week at room temperature (RT). Nevertheless, in order to be able to do RNA isolation in the bench, we do not use it.
16. From now on, all the steps will be performed at RT since temperatures below 20 °C can lead to some components of the buffer used to precipitate, impacting samples' purity.
17. Ethanol has to be diluted in DEPC-treated water (0.1 % DEPC in MilliQ water. Let DEPC act for 30 minutes at RT. Mix a few times during the incubation. Finally, autoclave for 20 minutes at 121 °C to decompose DEPC). The ethanol dilution should be done fresh, since there is a tendency to absorb water from the environment and, thus, get more diluted than pretended.



18. Note that the volume to elute RNA can be lower or higher depending on the concentration of cells used initially. Nevertheless, it should not be less than 50  $\mu\text{L}$  or higher than 100  $\mu\text{L}$ , as recommended by the manufacturer.
19. Almost all the kits used for RNA extraction are very specific for RNA isolation. However, genomic DNA can be co-purified and has to be degraded, or removed, to avoid misleading results. There are several different ways to get rid of genomic DNA. Here we use a DNase to degrade gDNA, and then the enzyme is inactivated by heat.
20. DNase is inactivated by heat, and since RNA is susceptible to degradation by heat in the presence of divalent cations (such as  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ ), EDTA - a chelating agent - is added to protect RNA from chemical degradation.
21. One can prepare a no-RT control by taking a small aliquot of the Xpert RTase and inactivate the enzyme by incubating at 85  $^{\circ}\text{C}$  for 5-10 minutes, prior to adding in step 3.
22. The cDNA dilution has to be optimized. It will depend for example on the amount of converted RNA, bacterial host, and Reverse Transcriptase used.
23. In order to minimize risk of contamination, reagent loss, and improve pipetting accuracy we recommend to prepare a mastermix for multiple samples (N), always including a negative control for the detection of possible contaminants. In order to do so, mix all components (N+1), except template DNA, dividing the mixture equally into each tube and then add template DNA (or PCR grade water in case of the control) to the individual PCR tubes or wells of a PCR plate.
24. When handling Xpert Fast SYBR (uni) minimize exposure to direct light, as exposure for an extended period of time might result in loss of signal intensity. Always certify that the product has been fully thawed and mixed well before use.
25. Primer design and purification is of the utmost importance, especially in case of low-copy number target detection, to minimize non-specific amplification with resulting loss of sensitivity. Primers should have melting temperatures of approximately 60  $^{\circ}\text{C}$ . To optimize results, use the lowest primer concentration that does not compromise the reaction efficiency (50-400 nM).
26. For efficient qPCR, under fast cycling conditions, it is recommended to amplify DNA fragments ranging from 80-200 bp. The shorter the amplicon, the faster the reaction can be cycled. Amplicons should not exceed 400 bp. High concentrations of template may inhibit PCR, result in non-specific primer binding, increased background fluorescence, and/or reduced linearity of standard curves. Results may be improved by using less template, and it is recommended to try a serial dilution to find the best concentration. It should be taken into consideration that the key factor is target copy number, and not the total amount of DNA. E.g.: 1  $\mu\text{g}$  of human genomic DNA might contain some 200,000 copies, whereas the same amount of bacterial DNA might contain 200 million copies. For small molecules, such as cDNA, 1 pg should result in a Ct around 20, whereas in order to obtain Ct of around 20 for human genomic DNA some 50 ng would be required. If copy numbers are really low (<100), primers are more likely to form primer dimers.
27. To monitor the reaction specificity and primer dimer formation, end-products must always be analyzed by melting curves.
28. Data analysis must be based on at least three replicates of three independent experiments.
29. Using unstained bacteria will define the bacterial population on FSC vs. SSC dot plot, drawing a gate around bacterial cells. This gating will allow to eliminate electronic background and/or debris interference. Individual FSC and SSC histograms should be analyzed to guarantee that the bell-shaped populations are



not cut off on the display. Peak shapes and resolution from noise will vary with bacterial morphology and sample matrix. The gate will vary with bacterial morphology and sample matrix.

30. When the emission spectra of different fluorochromes overlap, the fluorescence derived from more than one fluorochrome may be detected. To correct for this phenomenon, fluorescence compensation might be used. It is important to analyze single-stained samples to guarantee that the fluorescence detected in a particular detector is derived from the fluorochrome that is being measured (e.g. SYBR green-stained bacteria should be FL-1 (SYBR) positive/FL-4 (PI) negative, and PI-stained bacteria FL-1 (SYBR) negative/FL-4 (PI) positive).
31. The time of incubation vary with bacterial morphology and sample matrix (e.g. 5–10 min for *Staphylococcus epidermidis*; 20 min for *Pseudomonas aeruginosa*).
32. Most flow cytometers cannot directly provide the cell concentration or absolute count of cells in a sample. In those cases, cell counting beads should be used.

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