Lab

Deng

Phages against Bacteria

PRACTICAL COURSE

03/03/2025 - 07/03/2025

Introduction

Bacteriophages, or colloquially known as phages, are one of the most abundant biological entities on earth – there are approximately 10^{31} phage particles in the biosphere[1]. They were officially discovered in the early 20th century and named bacteriophages, which means 'bacteria eater', due to their ability to kill bacteria[2,3]. Indeed, these phages were used immediately, with some success, to treat bacterial diseases. This use of phages for combating bacterial infections is known as phage therapy. However, after the discovery of penicillin in 1928, humanity entered the 'Golden Age' of antibiotics and phage therapy was largely ignored for decades[1,4].

Throughout the years, bacteria are increasingly developing resistance to available antibiotics, with only a few new antibiotics being discovered. This has led to a global crisis known as antimicrobial resistance, which is predicted to cause 10 million deaths annually by the year 2050 onwards[5]. Phage therapy is therefore explored as a strategy to treat infections caused by antibiotic-resistant bacteria. Furthermore, due to phages being host-specific, phage therapy can target pathogenic bacteria without affecting non-target bacteria (for example, those present in the human microbiome)[6,7]. In contrast, antibiotics tend to have a broader range of targets, often leading to non-specific killing, which in turn can disrupt the gut flora (i.e., causing dysbiosis)[8,9].

As mentioned above, an important feature of phages is their abundance in nature, existing in diverse environmental niches that their prokaryotic hosts inhabit, ranging from seawater to the human gut. In practical terms, this presents us with ample opportunities to isolate these phages for phage therapy.

Prior to application as phage therapy, isolated phages must be thoroughly characterized and subsequently selected based on certain criteria[4,10]:

1. **Phages must be efficient in killing their bacterial host.** Phage efficacy is the measure of the extent of killing that phages are capable of inducing on their bacterial hosts. In the simplest term, an efficient phage needs to eliminate a bacterial population at a rapid rate with minimal resistance. This can be measured using growth kinetics of the bacteria in the presence of different concentrations of the phage.
2. **Phages must be lytic and not temperate**. In general, phages can be categorized as virulent (strictly lytic) or temperate. This categorization is based on their lifestyles when infecting the bacterial host: lytic or lysogenic cycles – to note, other lifestyles also exist, namely chronic and pseudolysogeny (see Phage Glossary below). Lytic phages typically pursue a single developmental program, in which they inject their DNA to the bacterial host, hijack the host machinery to replicate and induce lysis of the host to release progeny virions. On the other hand, temperate phages are capable of undergoing both lytic and lysogenic

cycles. In lysogenic cycle, the phage DNA is incorporated into the bacterial genome and replicated alongside the host indefinitely without killing it. Once integrated into the bacterial genome, the phage is known as prophage, which may carry virulent factors like toxins that are beneficial for the bacterial host. In summary, temperate phages are not suitable for phage therapy due to their lower efficacy in killing bacteria and also their potential for spreading virulent factors via horizontal gene transfer.

Last but not least, while *in vitro* tests can reveal various information about the interactions between phages and bacteria, they lack complexity to adequately represent immune responses to phage therapy. Thus, before phage therapy is administered to human patients, its safety and efficacy need to be tested in an animal model. An example of available animal models is the larvae of greater wax moth (*Galleria mellonella*). There are multiple reasons for why this invertebrate is an attractive model for studying bacterial infections and novel antimicrobial treatments[11]. First, they lack nociceptors and thus they are insensitive to pain, easing ethical rules unlike for vertebrate models. Second, although lacking adaptive immune system, invertebrates have similar innate immune responses to their vertebrate counterparts – including mammals – thanks to an extensive number of orthologous genes that control general functions. To note, the lack of adaptive immunity in invertebrates (absence of T- or B-lymphocytes and no production of antibody) has been construed as a limitation of this group of animals for studying infections. Albeit, evidence suggests that *G. mellonella* possess an immune-like memory against foreign microorganisms, known as larval-trained immunity, which is mediated by hemocytes and antimicrobial peptides. Furthermore, during infection processes, soluble effectors in *G. mellonella* bind to the bacterial lipopolysaccharide or lipoteichoic acid, triggering a cascade of reactions that leads to the production of melanin. Thus, infected *G. mellonella* larvae can be easily identified by their color change (melanization)[11]. Additionally, *G. mellonella* larvae offer a cost-effective and ethically sound alternative for testing phage therapy; their small size and low cost enable large-scale screening studies, aiding in the development of novel antimicrobial treatments while addressing ethical and practical considerations. Furthermore, the wax moth larvae have a short lifecycle, allowing for rapid experimentation and data collection, accelerating the pace of research.

In this course, we will explore the three main steps involved in the development of phage therapy: (1) phage isolation and characterization; (2) *in vitro* testing of phage efficacy and phage-antibiotic synergy; (3) *in vivo* testing of phage in the animal model *G. mellonella*; (4) phage genome assembly and annotation.

References

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Experimental Set up

Experiment 1: Isolating and Characterizing Phages

Phages are the most abundant entity on earth. They are present in various clinical and environmental samples, that when designing phage therapy experiments to combat specific bacterial infections, it makes the most sense to screen for the presence of phages where the bacterial hosts naturally exist. The sample (wastewater, soil, fecal sample, blood, saliva, sputum, etc.) is processed to remove bigger particles such as environmental waste, cell debris and bacteria then the Virus Like Particles (VLPs) are isolated from the resulting supernatant and quantified using plaque assay, which is a fundamental experiment to enrich and quantify bacterial viruses, the principle of which relies on overlaying a mixture of bacteria and phages on a solid agar medium, the bacterial growth would form a lawn and if the phage can lyse the bacterial host, then zones of lysis that result from the burst of phage progeny and lysis of the bacteria will form. These lysis zones are called plaques and the infectious phage unit is called a plaque forming unit (PFU). Plaque morphology is the first step into characterizing newly isolated phages, one can differentiate between virulent and temperate phages based on how the plaques they form look like on the plate: clear versus turbid lysis, size of plaque, presence/absence of a halo…

In this experiment you will get to process your own samples (for example, lake water) and wastewater samples to screen them for phages. Then, you will characterize two phages infecting a *Pseudomonas aeruginosa* strain based on their plaque morphology and growth kinetics.

Experiment 2: Comparing the antibacterial efficacy of phages and antibiotics

Phage therapy is considered a potential approach for treatment of the increasing number of antibiotic-resistant pathogens. Yet, it is important to explore the potential of combination therapies involving antibiotics and phages as an alternative for treating infections with multidrug-resistant bacteria. Phages and antibiotics can employ selective pressure on bacteria, which can compel them towards gene mutations. Under this selective pressure, there is loss or down-regulation of some of the host bacteria's genes related to toxicity, drug sensitivity and growth factors. This phenomenon is called an evolutionary trade-off where bacteria are faced with the choice to either develop resistance to antibiotics or to phages; for example, mutations that confer resistance to phages may also alter bacterial cell surface structures, making them more susceptible to antibiotics. On the other hand, mutations that provide resistance to antibiotics may increase bacterial susceptibility to phage infection.

In this experiment, you will assess the efficacies of the *Pseudomonas* phages characterized in Experiment 1. Additionally, you will investigate the potential synergy

between phages and antibiotics for killing *P. aeruginosa*. The main parameter that you will monitor is bacterial growth in the presence of phages, antibiotics or both.

Experiment 3: *In vivo* phage therapy in *Galleria mellonella*

After isolation and characterization of virulent phages *in vitro*, the *in vivo* efficacy of phage therapy can be assessed through the model of *P. aeruginosa*-infected *G. mellonella* larvae. Phages can be administered to larvae either prophylactically (before infection) or therapeutically (after infection), and the survival rates and bacterial loads in infected larvae are measured. Here, the wax moth larvae serve as a simplified model for studying host-pathogen interactions and evaluating the efficacy of antimicrobial agents. While *G. mellonella* larvae do not reflect the complexity of the human immune system, they exhibit innate immune responses that share some similarities with aspects of the human immune system. These include phagocytosis by hemocytes (immune cells analogous to mammalian macrophages), production of antimicrobial peptides, and activation of melanization and coagulation cascades in response to microbial infection.

In this experiment, you will start by inoculating a bacterial suspension containing a pre-determined bacterial cell count to each larvae. Then, you will inject antimicrobial agents into your treatment condition (phage and/or antibiotics). After the incubation period, you will monitor the larvae's color, appearance and movement to determine the survival rate and success rate of phage therapy.

Experiment 4: Phage genome assembly and annotation

As only virulent phages can be used for phage therapy, each phage must be screened for genes indicating the capability of lysogenic lifestyle (for example, those encoding integrases). Furthermore, for safety reasons, phages must not carry toxin and/or virulent genes. Hence, genomic sequencing is an integral part of phage therapy development. Prior to clinical application – after preliminary *in vitro* selection (Experiments 1 and 2) – phages are typically subjected to whole genome sequencing. To this end, the contigs of the phage genome must be assembled and annotated prior to downstream bioinformatics analyses.

In this experiment, you will assemble and annotate the genome of phages T4 and T7, phages that replicate lytically in *Escherichia coli*. Using the gene annotation, you will identify the gene encoding the "head-tail" connector protein, followed by matching this gene with its corresponding protein sequence. Based on the protein sequence that you obtain, you can visualize the 3D structure of this protein – along with other proteins that you find interesting – on AlphaFold platform.

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Timeline

	Morning (09:00-12:00)	Lunch break (12:00-13:00)	Afternoon (13:00-17:00)
Day 1	• General introduction to phage therapy • Processing your samples (Lake/ Waste water)		• Enrich the virus like particles (VLPs) by incubating the samples with your target bacteria (*Escherichia coli* K12, *E. coli* H10407, *E. coli* 51, *E. coli* 52 or *E. coli* 54) • Quantify lytic *P. aeruginosa* phages with plaque assay • Overnight culture of *Pseudomonas aeruginosa*, *E. coli* K12, *E. coli* H10407, *E. coli* 51, *E. coli* 52 or *E. coli* 54
Day 2	• Process phage enrichment from lake and WW samples and perform spot assays to screen for phages against your target bacteria (*E. coli* K12, *E. coli* H10407, *E. coli* 51, *E. coli* 52 or *E. coli* 54) • Check on your plaque assay plates with *P. aeruginosa* from yesterday and quantify the phages by counting plaques (record the number of plaques) • Conduct spot assay using *P. aeruginosa* phages against your *E. coli* bacteria		• Perform tests to determine the Minimal inhibitory concentrations (MIC) for antibiotics against *P. aeruginosa* • Perform spot assay using *P. aeruginosa* phages against *P. aeruginosa* bacteria • Overnight culture of *P. aeruginosa* • Finish at 15:00
Day 3	• Presentation of paper 1 • Presentation of paper 2 • Check on spot assays from your lake and WW samples enrichments and determine if you've isolated any novel phages! • Record results of MIC		• Perform growth kinetics of your bacteria in presence of antibiotics versus phages using the plate reader • Overnight culture of *P. aeruginosa*
Day 4	• Presentation of paper 3 • Presentation of paper 4 • Presentation of paper 5		• Check the growth kinetics and save on a USB stick • Select larval candidates and prepare stocks of antibiotics and phages • Perform in vivo phage therapy with *P. aeruginosa*-infected *G. mellonella* larvae
Day 5	• Bioinformatics (online)		• Analyze *in vitro* growth kinetics and *in vivo* larval survival results (only one representative of each group needs to be present to take photos and assess activity of the larvae, but everyone is welcome to join) • General discussion of the practical course results (online and in-person)

Seminar papers

Paper 1: Metagenome Data on Intestinal Phage-Bacteria Associations Aids the Development of Phage Therapy against Pathobionts

Paper 2: Parallel evolution of *Pseudomonas aeruginosa* phage resistance and virulence loss in response to phage treatment *in vivo* and *in vitro*

Paper 3: Phage Paride can kill dormant antibiotic tolerant cells of *Pseudomonas aeruginosa* by direct lytic replication

Paper 4: Phage-antibiotic combination therapy against drug-resistant *Pseudomonas aeruginosa* infection to allow liver transplantation in a toddler

Paper 5: Enhancing bacteriophage therapeutics through *in situ* production and release of heterologous antimicrobial effectors

Experiment 1: Isolating and Characterizing Phages

Processing of Lake water/Wastewater Samples (Day 1)

Fecal, lake water and wastewater samples are a good source of phages, but they're also full of bacteria and many other things that we must remove before we can screen for phages.

1. Transfer 20 mL of your sample and the wastewater into a 50 mL Falcon. Centrifuge for 10 minutes at 700 x g, transfer the supernatant into a fresh falcon tube and briefly vortex.
   → This is to ensure that all organic (fecal) matter is separated from bacteria and virus like particles (VLPs)

2. Centrifuge for 45 minutes at 6000 x g.
   → This is to pellet the bacteria, leaving the VLPs in the supernatant

3. Using a syringe and a syringe filter, filter supernatant through a 0.45 µm filter.

Liquid Enrichment of Unknown Phages from Sample (Day 1 & 2)

To see if any of the phages present in a sample (such as your samples and wastewater) are able to infect our target bacteria, we must add these bacteria and allow any phages which can infect it to replicate. Every group will get a different *E. coli* strain (K12, H10407, 51, 54, 55) to try isolating phages for.

1. Add 10 mL of prepared sample to 10 mL of 2 x LB in a 250 mL Erlenmeyer flask.

2. Add 2 mL of an overnight culture of your *E. coli* strain.
   → These volumes can be scaled up or down as required – but you should always use a flask much bigger than the volume of LB you are using.

3. Incubate shaking at 37 °C overnight.

4. Prepare overnight cultures of your *E. coli* strains by adding 10 mL of LB into a 50 mL Falcon and then add one colony of your strain with an inoculation loop. Also incubate shaking at 37 °C overnight.

5. Next day, transfer the culture to 50 mL falcon tubes and centrifuge at 6000 x g for 15 minutes to pellet the bacteria.

6. Using a syringe and syringe filter, pass the supernatant through a 0.45 µm filter.

- → Some people prefer to use a 0.22 µm filter for this (and previous filtration steps). Whilst this will ensure the sample is completely free from bacteria, it may also exclude some very large phages.

7. Use a spot plate to test for the presence of phages in your prepared samples (see pages 10 and 11).

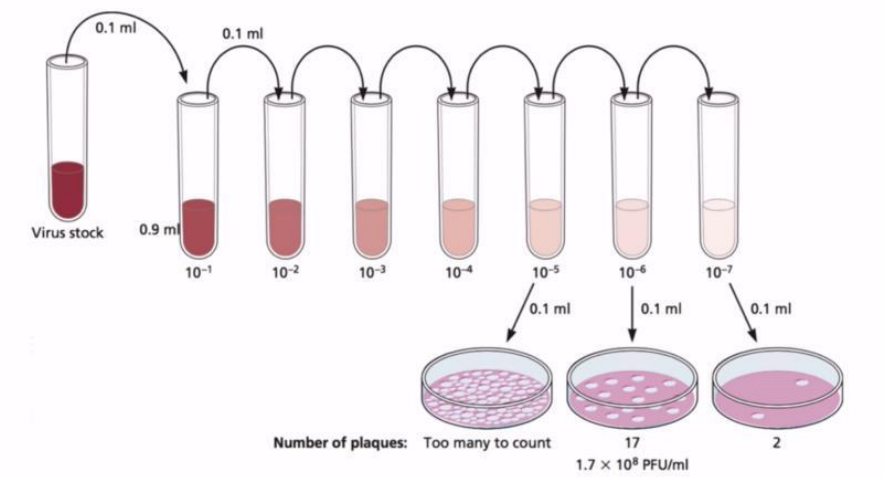
Plaque Assays

Plaque assays and spot plates are used to measure if there are phages present in a sample, and if so, how many? A plaque assay will give you a specific quantity (the phage titre) and a spot plate will give you a yes/no answer and an approximate idea of the titre.

Plaque Assay (Day 1, Quantification of two *P. aeruginosa* phages)

Preparation:

- → Turn the water bath on at a temperature of approx. 50 °C.
- → Plug the heat block in inside the hood (It is already set to a temperature of 45 – 48 °C, which we will need)
  - → High temperatures will make the process easier, but some bacteria require the lower temperature to grow
- → Microwave the soft agar until it is completely melted and put it into the water bath
  - → Make sure the lid is loose in the microwave
  - → The agar should sit in the water bath for long enough that the agar has cooled down a little – if it is used straight from the microwave it will be too hot and will kill the bacteria
- → Label plates and Eppis (Bacteria, dilution, replicate, date, group)
  - → 6 plates per plaque assay – 3 dilutions in duplicate, for 2 phages
- → Dilute your phages
  - → Do a 1:10 serial dilution.
  - → We are going to plate out 3 different dilutions. You want to plate the dilution which are going to give you between 30 and 300 plaques on the plate. You can work this out from the expected approximate titre, or if you're not sure, the 10^6, 10^7, and 10^8 dilutions are a good place to start



Procedure:

- → Add 3 mL of molten soft agar to a glass tube and place it in the heat block
    - → 1 for each plate
- → Add 100 µL of phage and 100 µL of bacterial overnight culture to each tube and vortex
    - → You can alter the amount of bacteria you use (it should be enough to make a lawn) but the amount of phage added must stay consistent
    - → Vortexing at this step is very important!
- → Pour the contents of the appropriate tube onto the labelled plate and swirl it around until the plate is fully covered.
    - → You have to move quickly at this stage so that the plate is fully covered before the agar sets
- → Incubate overnight at 37 °C
- → Count the plaques and calculate the phage titre using the following formula:

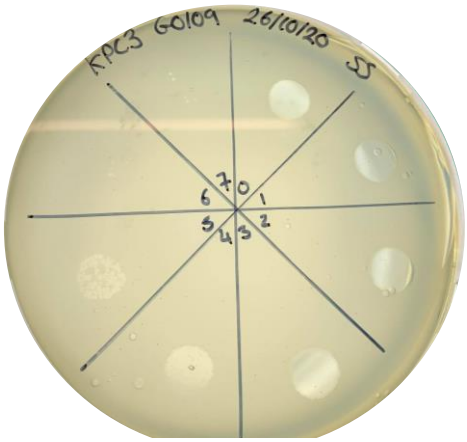
PFU/mL = no. of plaques x dilution factor x 10

- → The extra x 10 is to account for the 'extra dilution' you do when you add 100 µL into the soft agar

Spot Plates (Day 2, Check for isolation of phages against *E. coli*)

Preparation:

- → Prepare as for the plaque assay – only the labelling on the plates will be different
    - → 1 plate per sample (so 2 plates per group)
    - → Your plate should be labelled for each of your dilutions (e.g. 0-7). Here is an example of how you could set up your plate:



Procedure:

- → Add 3 mL of molten soft agar to a glass tube and place it in the heat block
  - → 1 for each plate
- → Add approx. 100 µL of bacterial overnight culture to each tube and vortex
  - → You can alter the amount of bacteria you use but it should be enough to make a lawn
  - → Vortexing at this step is very important!
- → Pour the contents of the appropriate tube onto the labelled plate and swirl it around until the plate is fully covered.
  - → You have to move quickly at this stage so that the plate is fully covered before the agar sets
- → Once the agar is set, spot 3 µL of each of your phage dilutions directly on top of the agar
  - → Make sure where you are spotting each dilution matches with the way you have labelled your plate
- → Incubate overnight at 37 °C
- → If there are phages present, single plaques should be visible in at least one of the dilutions. Document your findings and take pictures of the plates.
  - → Sometimes, a phage which is not specific for a host can still kill the host at a high enough concentration. If you cannot see individual plaques, you cannot conclude definitively that the phage infects the bacteria. Why?
- → Repeat the same process for testing *P. aeruginosa* phages against *E. coli* host

Experiment 2: Comparing the antibacterial efficacy of phages and antibiotics

Antibiotic versus Phage Susceptibility

You will determine the minimal inhibitory concentrations of select antibiotics and your previously characterized *P. aeruginosa* phages. This is to assess the minimal amount of each antibacterial component necessary for sufficient inhibition of bacterial growth. The experimental setup is similar to spot assays.

Procedure:

- → Add 3 mL of molten soft agar to a glass tube and place it in the heat block
    - → 1 for each plate
- → Add approx. 100 µL of bacterial overnight culture to each tube and vortex
    - → You can alter the amount of bacteria you use but it should be enough to make a lawn
    - → Vortexing at this step is very important!
- → Pour the contents of the appropriate tube onto the labelled plate and swirl it around until the plate is fully covered.
    - → You have to move quickly at this stage so that the plate is fully covered before the agar sets
- → Once the agar is set after 3-5 minutes:

For antibiotics:

- → Place the respective antibiotic test strips using tweezers on the middle of the plate containing the solidified soft agar
    - → The antibiotic strip contains a gradient of antibiotic concentrations along its length. This gradient ranges from high concentrations at one end to low concentrations at the other end
    - → During incubation as appropriate temperatures, the antibiotic diffuses outward from the strip into the surrounding agar and inhibits bacterial growth around the strip, forming an elliptical zone of inhibition.
    - → The edge of this zone, where bacterial growth is visibly inhibited, corresponds to the minimum inhibitory concentration (MIC) of the antibiotic for the bacterial strain being tested
- → Incubate plates overnight at 37 °C

For phages:

→ Spot 3 µL of each of your phage dilutions directly on top of the agar

→ Make sure where you are spotting each dilution matches with the way you have labelled your plate

→ Incubate plates overnight at 37 °C

Plate Reader Kinetics

A plate reader is a machine that measures the OD_{600} of samples in a 96 well plate at regular intervals over a set time period (for example, every 20 minutes for 24 hours). This allows us to monitor how well the bacteria is growing and compare how it behaves on its own compared to with the phage.

→ Discussion point: what additional information can we infer from observing continuous bacterial growth in the presence of inhibitory compounds as opposed to simply incubating the culture overnight?

→ Discussion point: what are we expecting to see in each treatment condition?

1. Inoculate a single colony of your bacteria of interest into 5 mL LB and incubate overnight at the appropriate temperature.

   → Each group will be responsible for the growth kinetics of a single bacterial colony in presence of either phages or antibiotics or both.

   → Discussion point: are we expecting to see differences in the effects of antibacterial compounds on individual colonies? What about individual bacterial clones? Why yes or why not?

2. Measure the OD_{600} of the bacterial overnight culture using the plate reader. Dilute until it is at OD_{600} of 0.45. Further dilute until the bacterial concentration around 10^6 CFU/mL. Calculate the volume of bacterial culture that corresponds to 10^5 CFU. Please use Table 1 to record this volume.

   → OD_{600} = 0.45 corresponds to a concentration of 2.79 x 10^9 CFU/mL.

3. Calculate the volume of phages you need to add to the bacteria to get the appropriate multiplicity of infection (MOI). Please use Table 1 to record these volumes

   → The multiplicity of infection or MOI refers to the ratio of infectious phage particles to the number of host bacterial cells in a given population. It is important to consider the MOI of any phage when designing experiments for phage therapy. The MOI is influenced by the infection efficiency, adsorption rate and burst size of the infectious phage.

   → For the phage dilutions, you will need to already know the titre.

4. Calculate the volume of antibiotics to add from the stock to correspond to the MIC of 1. Please use Table 1 to record these volumes.

5. Pipette the following samples into the 96-well plate (see layout on Table 2)
    - Bacteria + Phage 1 (MOI 1)
    - Bacteria + Phage 1 (MOI 10)
    - Bacteria + Phage 2 (MOI 1)
    - Bacteria + Phage 2 (MOI 10)
    - Bacteria + Phage 1 + Phage 2 (MOI 1)
    - Bacteria + Phage 1 + Phage 2 (MOI 10)
    - Bacteria + Antibiotic 1 (1*MIC)
    - Bacteria + Antibiotic 2 (1*MIC)
    - Bacteria + Phage 1 + Antibiotic 1 (MOI1, 1*MIC)
    - Bacteria + Phage 1 + Antibiotic 2 (MOI10, 1*MIC)
    - Bacteria + Phage 2 + Antibiotic 1 (MOI1, 1*MIC)
    - Bacteria + Phage 2 + Antibiotic 2 (MOI10, 1*MIC)
    - Positive control: Bacteria only
    - Negative controls: Phage 1 only, Phage 2 only, LB medium.

6. Set the program in the plate reader

    → Set the temperature to 37 °C, double orbital shaking with measurements at OD_{600} every 20 min for 24 h

    → Discussion point: what are you expecting to see in the growth kinetics results for the different treatment groups?

Table 1. Volume of each component in a reaction. Please ensure you **add the bacteria last!**

	B	P1	P2	A1	A2	LB	Total
B + P1 (MOI 1)			X	X	X		400
B + P1 (MOI 10)			X	X	X		400
B + P2 (MOI 1)		X		X	X		400
B + P2 (MOI 10)		X		X	X		400
B + P1 + P2 (MOI 1)				X	X		400
B + P1 + P2 (MOI 1)				X	X		400
B + A1 (1*MIC)		X	X		X		400
B + A2 (1*MIC)		X	X	X			400
B + P1 + A1 (MOI1, 1*MIC)			X		X		400
B + P1 + A2 (MOI10, 1*MIC)			X	X			400
B + P2 + A1 (MOI1, 1*MIC)		X			X		400
B + P2 + A2 (MOI10, 1*MIC)		X		X			400
B		X	X	X	X		400
P1	X		X	X	X		400
P2	X	X		X	X		400
LB	X	X	X	X	X		400

Table 2. Plate layout for bacterial kinetics in the presence of phages and/or antibiotics.

		Group 1			Group 2			Group 3			Group 4		
		MOI 1	MOI10	Controls	MOI 1	MOI10	Controls	MOI 1	MOI10	Controls	MOI 1	MOI10	Controls
		1	2	3	4	5	6	7	8	9	10	11	12
1* MIC	A	B+P1	B+P1	B	B+P1	B+P1	B	B+P1	B+P1	B	B+P1	B+P1	B
	B	B+P2	B+P2	P1	B+P2	B+P2	P1	B+P2	B+P2	P1	B+P2	B+P2	P1
	C	B+P1+P2	B+P1+P2	P2	B+P1+P2	B+P1+P2	P2	B+P1+P2	B+P1+P2	P2	B+P1+P2	B+P1+P2	P2
	D	B+P1+A1	B+P1+A1	B+A1	B+P1+A1	B+P1+A1	B+A1	B+P1+A1	B+P1+A1	B+A1	B+P1+A1	B+P1+A1	B+A1
	E	B+P1+A2	B+P1+A2	B+A2	B+P1+A2	B+P1+A2	B+A2	B+P1+A2	B+P1+A2	B+A2	B+P1+A2	B+P1+A2	B+A2
	F												
	G												
	H	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB	LB

Experiment 3: *In vivo* phage therapy in *Galleria mellonella*

Efficacy of phage therapy in a larvae model against *P. aeruginosa*

1. Make an overnight culture of *P. aeruginosa* by inoculating a single colony into 5 mL of LB and incubate it overnight at the appropriate conditions.
2. Grow a day culture from your overnight culture by inoculating 50 µL of overnight culture into 5 mL of LB, incubate the culture until the OD_{600} reaches 0.1
3. Harvest the bacteria by centrifugation at 6000 x g for 10 minutes.
4. Wash the bacteria twice with PBS buffer and dilute it to a final concentration of 5×10^5 CFU/mL.
5. Dilute both your phages in SM buffer to a final concentration of 5×10^5 PFU/mL (corresponding to an MOI of 1)
6. Select 30 larvae of similar weight with no signs of pigmentation

    → You need to use 5 larvae per condition:
        - Bacteria alone
        - Phage alone
        - Bacteria + phage (PT)
        - Bacteria + antibiotic
        - Bacteria + phage + antibiotic
        - No injection control

    → Discussion point: What purpose does each control/condition serve?

7. Surface-sterilize each larva with a 70% ethanol rinsed cotton swab
8. Inject 10 µL of the bacterial suspension in the last left proleg in the larvae that will contain bacteria (bacteria alone + bacteria and phages)

    → Inject the 10 µL of SM buffer to the control group

9. Incubate for 1 h at 37 °C

    → Incubate all larvae, even the ones with no injection yet to stay consistent

10. Inject 10 µL of phage suspension in the last right proleg in the phage therapy larvae group and the phage alone control
11. Incubate all your larvae at 37 °C in the dark overnight
12. The next day, count the number of larvae that show any level of pigmentation

    → Score the success of your phage therapy on larvae using the scheme in Table 3

    → Make sure to document all your results with pictures

Table 3. Scoring scheme for determining the extend of infection in *Galleria mellonella*. Taken from van Nieuwenhuyse et al. (2022).

Category	Description	Score
Activity	No activity despite stimulation	0
	Minimal activity upon stimulation	1
	Normal activity upon stimulation	2
	Spontaneously active without stimulation	3
Melanization	Complete melanization: black larvae	0
	Brown larvae with darker spots	1
	Spots on beige larvae	2
	<3 spots on beige larvae	3
	No melanization: cream white larvae	4
Survival	Dead	0
	Alive	2
Total	Sum of all categories (0-9)	

Experiment 4: Phage genome assembly and annotation

Prerequisites

1. Create an account in https://usegalaxy.eu
2. Create an account in https://alphafoldserver.com/
3. Download the four .fastq files for T4 and T7 bacteriophages in this link:

Phage genome assembly

1. Sign in to your galaxy account https://usegalaxy.eu.
2. Choose the "upload" from the menu on the left side, input the two T7 bacteriophage .fastq files that were downloaded previously.
3. Set the "file type", the second column, to fastq.
4. Click "start" and wait until the upload is complete.
5. From the left menu, select the "Tools" section and search for "metaSPAdes".
6. In the "FASTQ file(s): forward reads" and "FASTQ file(s): reverse reads" part, press the "Select Values" and choose the R1 (Forward) and R2 (Reverse) fastq files, respectively.
7. Further down, from the "Set Phred quality offset" select "33 (sanger)".
8. Finally press "Run Tool" at the bottom or top right side.
9. A green message should appear with the title of "Started tool metaSPAdes and successfully added 1 job to the queue." which indicates a successful start.
10. After successful completion, the job field on the right side will also turn green and we are ready for the annotation step.

    → What is the length on the assembled genome? Is it the same length as the genome submitted in the NCBI database?

11. Download the assembled genome (which is called scaffold.fasta in this case) and visually inspect it using a text editor.

Phage genome annotation

1. From the left menu, select the "Tools" section and search for "Pharokka" for phage genome annotation or the following link: https://usegalaxy.eu/?tool_id=toolshed.g2.bx.psu.edu%2Frepos%2Fiuc%2Fpharokka%2Fpharokka%2F1.2.1%20galaxy0.
2. In the "Bacteriophage genome" section, choose the "metaSPAdes on data # and data #: Contigs"
3. Switch the "Create a Zip archive of the complete output for further investigation."
4. From the right menu select the "pharokka on data #: Genbank" and "pharokka on data #: zip of the complete output" to download the annotation results.

- → What information is stored in a Genbank file?
- → Unzip the pharokka results and from the "pharokka_cds_functions.tsv" file find out how many proteins are involved in the "lysis" process. Could you find those CDS in the "pharokka_cds_final_merged_output.tsv" file?

5. Upload the Genbank file (.gbk) to proksee website for visualization of annotation:
   https://proksee.ca/

   → How is the phage genome organized? How many blocks of ORFs can you find?

6. Find the "head-tail" connector protein from the "pharokka_cds_final_merged_output.tsv" and use its corresponding gene ID to find the protein sequence from the "phanotate.faa" file.

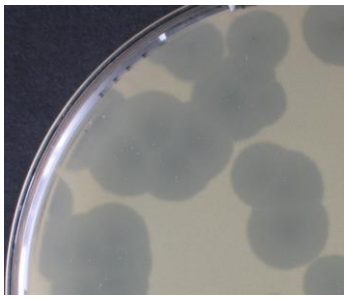
   → The "head-tail" connector protein is located on the positive or negative strand of the genome?

7. Go to https://alphafoldserver.com/ (you will have to sign in with a Google account) and paste in the protein sequence from step 3. After a short waiting time, you should be able to look at the predicted 3D structure of the head-tail connector protein. Feel free to repeat the same step for other proteins you find interesting.

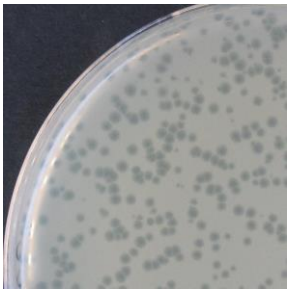
Phage Glossary

Plaque

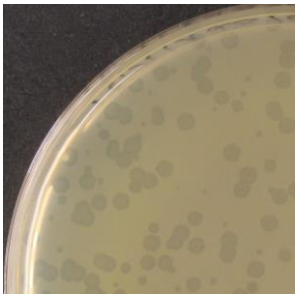
A plaque is the small zone of bacterial lysis that is observed when a phage infects a bacteria in a plaque assay – it is the phage equivalent of a bacterial colony. The morphology of plaques can vary, and you may be able to tell some information about the phage from this – larger phages tend to have smaller plaques, and vice versa, phages encoding depolymerases will have halos, and temperate phages tend to have turbid plaques.



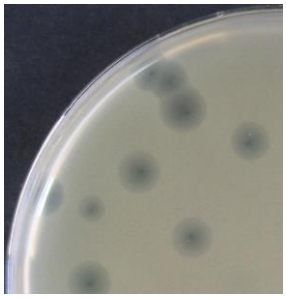
Large plaques with a halo



Small plaques



Turbid plaques



Medium plaques with a halo

MOI

Multiplicity Of Infection – this is the ratio of phages to bacteria. An MOI of 1 would mean there were 1 phage for every bacteria, an MOI of 10 would mean 10 phage for every bacteria, and an MOI of 0.1 would be 10 bacteria for every phage.

Virulent versus temperate phages

A virulent phage is only able to undergo the lytic lifecycle, whereas a temperate phage can undergo the lytic or the lysogenic life cycle.

Lytic cycle

The lytic life cycle involves the phage infecting the bacteria, replicating itself and then lysing the bacteria, releasing progeny virions.

Lysogenic cycle

In the lysogenic life cycle, the phage incorporates itself into the bacterial genome as a prophage where it can stay indefinitely.

Chronic cycle

This is a non-bactericidal infection step, in which virions are continuously synthesized and extruded from the bacterial host cell, typically occurring in the viral family *Inoviridae* (filamentous phage).

Pseudolysogenic cycle

The stage of stalled development of a phage in a host cell without either multiplication of the phage genome (as in lytic cycle) or its replication synchronized with the cell cycle and stable maintenance in the cell line (as in lysogenic cycle). This cycle typically occurs when the bacterial host is under unfavorable growth conditions.

CFU

Colony Forming Units – used to measure the titre of bacteria

PFU

Plaque Forming Units – used to measure the titre of phages

OD

Optical Density – a measure of how turbid a solution is, used as a proxy for how many bacteria are present. This is usually measured at a wavelength of 600 nm.

Writing and Submission of Report

Deadline and submission procedure

Each student needs to submit their own report, i.e., not as a group. Report must be submitted by **7 April 2025 at 23:59**. Please send your report as a PDF file via email to Prof. Dr. Li Deng at li.deng@tum.de.

If you have questions regarding the report, please contact Adrian Thaqi at adrian.thaqi@helmholtz-munich.de.

How to write your report

- No strict page limits, but do **not underwrite nor overwrite** (10-30 pages are recommended). Ensure that you include necessary information for the readers to understand your experiments, but do not overwhelm them with details. Imagine this is like writing a story about phage therapy. Have a coherent narrative.
- Your report should include the following sections:
    - Introduction
        - Keep it **concise**. In scientific writing, this is the part where you introduce your research to the audience. Imagine you are a phage biologist and you must justify why you are doing a research on phage therapy. So, find existing body of evidence around the topic of phage therapy, summarize it and create an interesting story. Consider the experiments that you did when writing the introduction, as you must keep it relevant. Include references where appropriate.
    - Methodology
        - When describing the methods, you do not need to go into specific details. Simply **demonstrate that you understand** why we used each method in the different experiments. For example, you can simply write "Plaque assay was used to quantify the number of infectious phages against a bacterial host. This was done by counting the number of zones of inhibition on the plate", instead of "In plaque assay, 100 µl of phage stock and 100 µL of bacterial culture were added into 3 mL of soft agar".
        - Make use of sub-sections to describe the different experiments.
    - Results and Discussion
        - You have a choice to write the results and discussion as "Results and Discussion" (combined into one section) or as "Results" and "Discussions" (two separate sections). Either way, please keep it **concise, relevant and descriptive**. Having said that, feel free to

be creative. If you encountered problems or observed something interesting during the experiments, you can suggest improvements or provide explanations. It goes without saying that your suggestions and explanations should be justified logically based on clear scientific evidence (include references where appropriate).

- Conclusion
  - Summarize what you did, what you found and improvements or explanations that you think are relevant. Do not repeat the description of your results in detail here. Keep this section short (150-250 words should be enough).

- Things to keep in mind when writing your report
  - Genus and species names should **always be italicized** (for example, *Escherichia coli* and not Escherichia coli).
  - Genus and species names should be written in full in the first mention (for example, *Pseudomonas aeruginosa*), with the genus being abbreviated in subsequent mentions (for example, *P. aeruginosa*).
  - In scientific writing, **avoid using subjective adjectives**. In other words, when describing something, provide a context.
    - Do not write "Phage therapy is a **very good** alternative to antibiotic treatments". Instead, write "Phage therapy is an alternative to antibiotic treatments".
    - Do not write "Phage A and B were found to be **better** than Phage C". Instead, write "The kinetics study showed that Phage A and Phage B had a higher efficacy in killing Bacteria A than Phage C". The first sentence lacks definition of the word "better" (i.e., better at what? Breakdancing, singing, or….?). In the second sentence, there is a context that explains how Phage A and B compared to Phage C.