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Fun with phages: how do heat and pH affect bacteriophage viability?

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Bacteriophages look like alien spaceships but they are actually viruses that infect bacteria. Use these fantastic beasts to explore protein stability.

Bacteriophages, also called phages, are the most-abundant organisms in nature. They are viruses that infect bacterial cells, ultimately leading to lysis of the cells and the release of new phage particles.

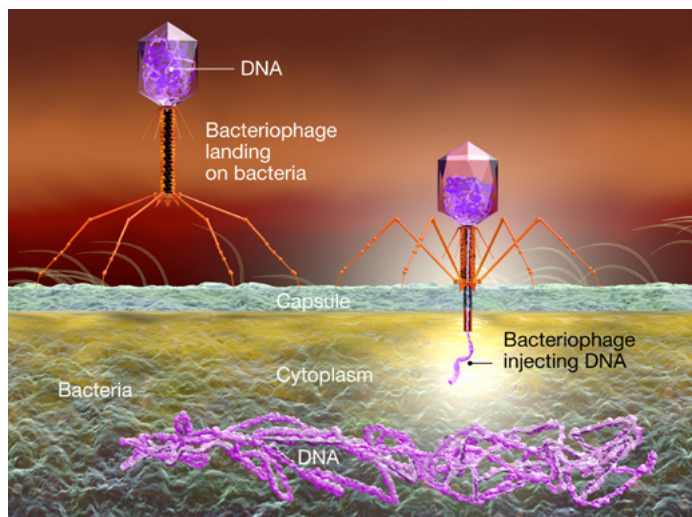


Figure 1. An artist's representation of T4 phage infecting a bacterium
Image: Anna-Lynn Wegener/Shutterstock.com

Two different activities have been devised using P22 phage (figure 1 and [background information](#)): one where the phages are subjected to different temperatures, and another where the phages are subjected to a solution of HCl at different pH values. Both activities are intended as aids to teach protein structure, but they employ different biological principles.

They can be used interchangeably if doing both is not possible, or they can both be implemented to teach different principles of protein biology or microbiology.

A suitable age range is about 16 to 18 years old.

Preparing the cultures

The phage stock solution can be prepared well ahead of time: see the [phage stock preparation](#) in the supporting material. Since phages are a kind of virus, they can't replicate alone; they need a bacterial host. In these activities, we use *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2, which is a nonpathogenic strain. *Salmonella enterica* is the binomial genus and species, Typhimurium is the serovar, and LT2 is the strain.

Note on phage/bacteria combinations

The procedure is based on *Salmonella enterica* bacteria and P22 phage; however, these experiments can also be done with *Escherichia coli* and T4 phage.

One to four days before doing the experiments, appropriate bacterial cultures need to be set up. This can be done by the students in an earlier lesson or simply prepared by the teacher or technician.



Safety notes

Bacterial liquid cultures should be treated with bleach before being discarded, and all micro-organisms should be discarded into appropriate waste containers.

Materials

- *Salmonella enterica* Typhimurium LT2 culture
- Lysogeny broth (LB; around 50 ml will be enough for both experiments)
- Miller's LB agar plate
- Inoculating loop or sterile cotton swab
- Sterile conical tubes or similar
- Bunsen/ethanol burner
- Sterile conical tubes or similar
- Pipettes and pipette tips

Procedure

Salmonella plate

1. Before the class (1–4 days), take a sample of frozen *Salmonella* preparation, and use an inoculating loop to streak it over the Miller's LB agar plate.
2. Incubate the plate at 37 °C overnight or at room temperature for 24–48 h (figure 2A). The same plate can be used for both activities.

Salmonella liquid culture

3. Either 3 h before the class or the night before, pipette 5 ml LB broth into a sterile conical tube, and then use the pipette tip to inoculate one colony from the *Salmonella* culture plate into it.
4. Incubate the tube with agitation at 37 °C for 3 h or at room temperature without agitation overnight (figure 2B).
5. From the liquid culture, create a 1/10 dilution using 9 ml LB broth and 1 ml of the liquid culture (figure 2B).

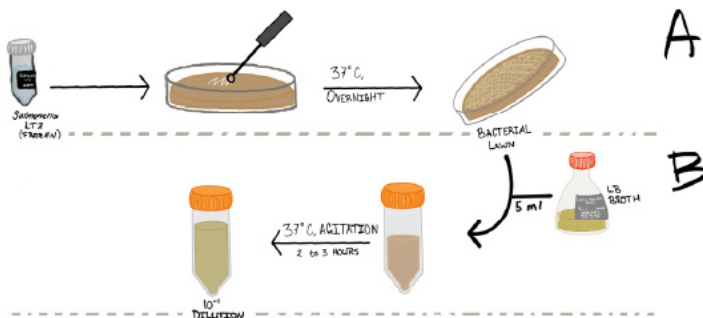


Figure 2. Step-by-step instructions on how to create the bacterial culture plate and the liquid cultures

Image courtesy of the author

Activity 1: Heat treatment of bacteriophages

Virtually all known bacteriophages (phages) are composed of a protein capsid and tail, including phage P22. This activity is designed to provide a visual aid to explain this structure, employing the principle of heat denaturation as a basis.

Phage P22 structure

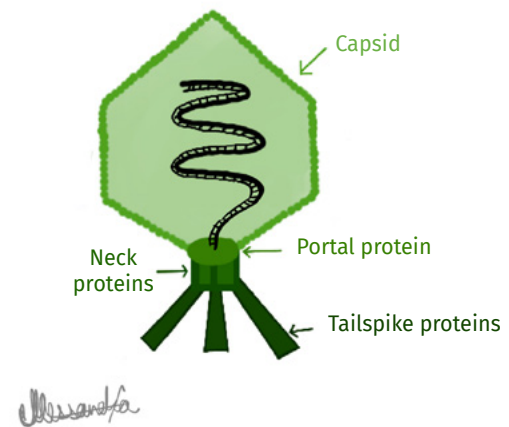


Figure 3. Drawing of the main proteins that make up *Lederbergvirus P22*
Image courtesy of the author

This activity takes two lessons to complete: one to set up the phage cultures, and a second to evaluate the results after incubation.



Safety notes

Bacterial liquid cultures should be treated with bleach before being discarded, and all micro-organisms should be discarded into appropriate waste containers.

Materials

Per student or group:

- [Bacteriophage infosheet](#)
- Thermostatic bath; alternatively, a pot of water and a thermostat
- Prepared *Salmonella enterica* Typhimurium LT2 cultures
- 100 µl *Lederbergvirus*/phage P22 stock
- 10 mM MgSO₄ solution (10 ml is enough for both experiments)
- TSA culture plates (1-3 per experimental condition)
- Liquid agar (3 ml per TSA plate)
- Sterile conical tubes or similar
- Bunsen/ethanol burner
- 1.5 ml Eppendorf tubes
- Pipettes and pipette tips

Procedure

1. Hand out the bacteriophage infosheet provided in the supporting material. Explain the structure and main features of phage biology to students before proceeding with the experiments. The students can also view the videos and articles in the resources section or do their own research.

Phage dilution and treatment

2. Next, create serial bacteriophage dilutions by taking the 100 μl of phage P22 stock and combining it, in a 1.5 ml Eppendorf tube, with 900 μl of MgSO_4 solution. From this first dilution, take 100 μl and combine it in another tube with 900 μl of MgSO_4 solution. Repeat twice more to achieve a 10^{-4} (1/10 000) dilution (figure 4).
3. Repeat this process to obtain four individual tubes of 10^{-4} dilutions (figure 4). Dilutions of 10^{-3} or 10^{-4} should be suitable, but this may need to be optimized. Ideally, teachers should try the experiment with their phage preparation beforehand, to check the appropriate dilution to give countable plaques.

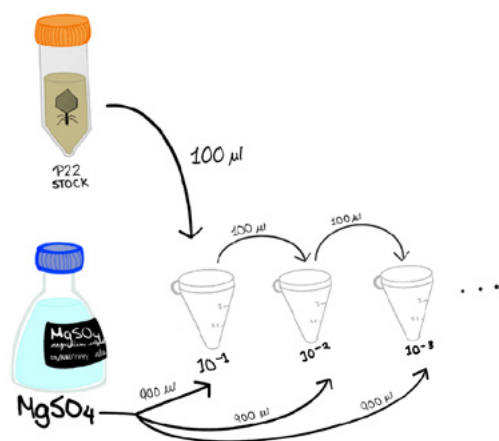


Figure 4. Step-by-step instructions on creating serial bacteriophage dilutions

Image courtesy of the author

4. Take three of the bacteriophage dilutions and treat them at temperatures higher than 50 °C (recommended: 50 °C, 75 °C, 100 °C) for 5–10 min; the untreated bacteriophage dilution serves as a control. Make sure you label the tubes with the temperature before you start heating.

Phage seeding

5. Use a permanent marker to label four TSA plates with the three conditions and control. Label the bottom of the plate and not the lid; lids can get mixed up.
6. Add 3 ml liquid agar to a 15 ml conical tube.
7. To this tube, add 100 μl of the 1/10 bacterial culture and 100 μl of the untreated 10^{-4} bacteriophage culture.

8. Shake by inversion, and pour the mixture over a TSA plate, ensuring it is distributed evenly (figure 5).

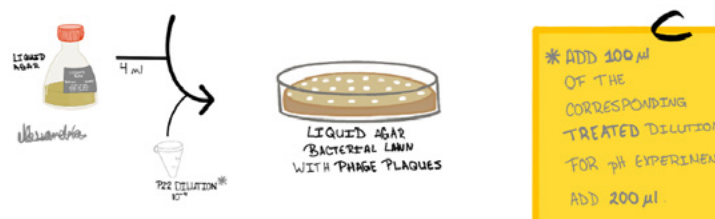


Figure 5. Phage seeding

Image courtesy of the author

9. Repeat step 6 for the treated phage dilutions; use one plate for each different condition (temperature). Use several plates for each condition if you want to do statistics, like calculating a mean and standard deviation (SD).
10. Once all the plates have the corresponding mixture, dispose of the bacterial liquid culture and the bacterial dilution by adding a bleach and mixing; dispose into an appropriate waste container.
11. Incubate the TSA plates at 37 °C overnight or at room temperature for two days.

Evaluation

12. After incubation, observe the plates. Is there anything about the plates that becomes more noticeable as the temperature increases?
13. Ask students to describe what they see. What do they think the clear patches (called plaques) are? Hint: T22 phages are viruses that infect bacteria and destroy them as part of their life cycle.
14. Having established that the plaques represent phages that infected bacteria and stopped them from multiplying at that point, what does the number of plaques say about the number of infective phages (plaque-forming units) in the sample?
15. Can students see a difference visually under different temperature conditions?
16. Optional: the plaques can be counted to create a graph as a visual representation, using the formula

Results and discussion

An example of expected results can be seen in figure 6. The control is not shown in this figure, but the incubation temperature can be used and plotted.

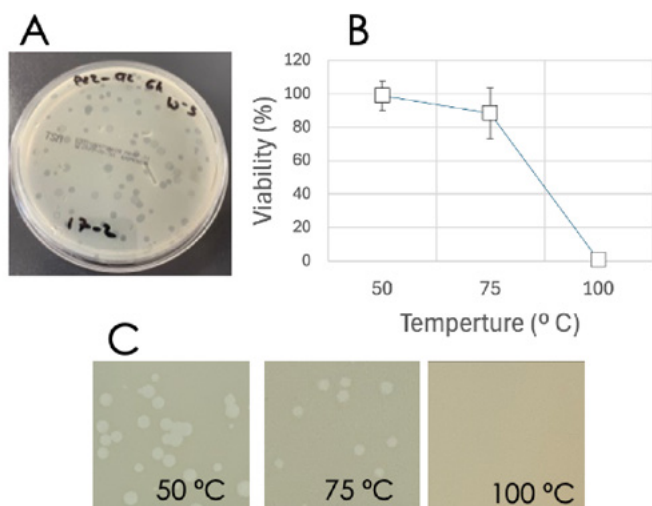


Figure 6. Expected results for temperature treatment. A) Image of the plaques obtained in TSA plaques. Effect of temperature on phage viability: B) graph showing the percentage of plaques referenced to an untreated control expressed in mean \pm SD; C) representative images of the plates, showing a decrease in the number of plaques.

Image courtesy of the author

Questions to aid the discussion:

- Knowing what you now know about bacteriophages, what do you believe this decrease in plaques with temperature is due to?
- Can you predict what would happen if we were to use higher temperatures?

Treating proteins at high temperature leads to a phenomenon known as protein denaturation, wherein a protein chain unfolds and the protein loses its 3D shape due to a loss of bonds within the protein. Bacteriophages, being made of proteins, are dependent on the environmental temperature to take on the correct form and be able to infect host cells. In the results, the student will observe how the number of plaques will decrease when the temperature rises, suggesting it is caused by protein denaturation.

Statistics can also be used if several plaques have been seeded for every treatment. The average and SD can be calculated and discussed with the students.

Activity 2: Acidic treatment of bacteriophages

In the next experiment, students can use phages to visualize the effects of suboptimal pH on protein stability.

The activity takes two lessons to complete: one to set up the phage cultures, and a second to evaluate the results after incubation. However, only the treatment of phages is different for the two activities, so the two experiments can be done simultaneously or separately.



Safety notes

Bacterial liquid cultures should be treated with bleach before being discarded, and all microorganisms should be discarded into appropriate waste containers.

Materials

- HCl (37%) or HCl solutions at the desired pH (here pH 2 and 4 have been used).
- pH strips
- 100 μ l Lederbergvirus/phage P22 stock
- MgSO₄ 10 mM solution (10 ml is enough for both experiments)
- TSA culture plates (one per experimental condition)
- Liquid agar (3 ml per TSA plate)
- Sterile conical tubes or similar
- Bunsen/ethanol burner
- 1.5 ml Eppendorf tubes
- Pipettes and pipette tips or measuring glasses

Procedure

1. Carry out the phage dilution as in steps 1–3 of Activity 1, but only three samples (not four) need to be prepared. Use one for the control and two for testing two different pH (recommended pH 2 and 4).
2. Instead of heating the samples, take a clean tube and place 100 μ l of the desired phage dilution. Add 100 μ l of the desired pH HCl solution, leaving it to act for 5 min. Repeat for the two pH conditions. Label the tubes before adding the acid.
3. Before adding the phages to the agar, use a piece of indicator paper to measure and record the pH of each sample.
4. Continue with phage seeding and incubation as per steps 5–9 in Activity 1, but use 200 μ l of the phage–HCl mix (step 2).
5. After incubation (37 °C overnight or at room temperature for two days), evaluate the plates and discuss the results as before.

Results and discussion

Expected results are shown in figure 7.

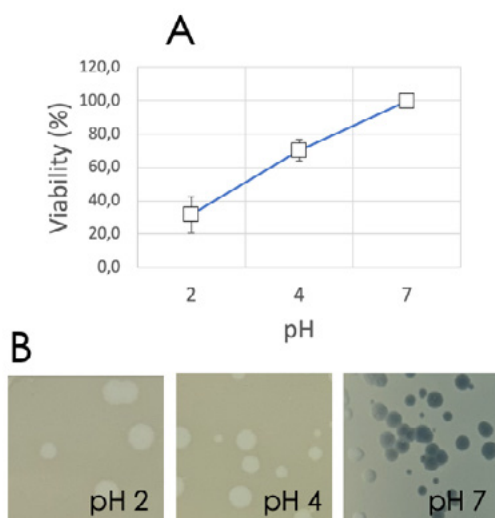


Figure 7. Expected results for the effect of pH on phage viability. A) Graph showing the percentage of plaques referenced to an untreated control expressed in mean \pm SD. B) Representative images of the plates obtained.

Image courtesy of the author

A decrease in the number of phage plaques can be observed as the pH value decreases, both in the images and in the graph. Proteins exist within a specific pH range: a pH level below or above this pH range will cause the amino acid residues to accept or release protons, which will change their charge and hydrophilicity. This prevents correct folding, which leads to a loss of phage viability. Thus, bacteriophages, being made of proteins, are dependent on the environmental pH to take on the correct structure and be able to enter host cells to replicate themselves.

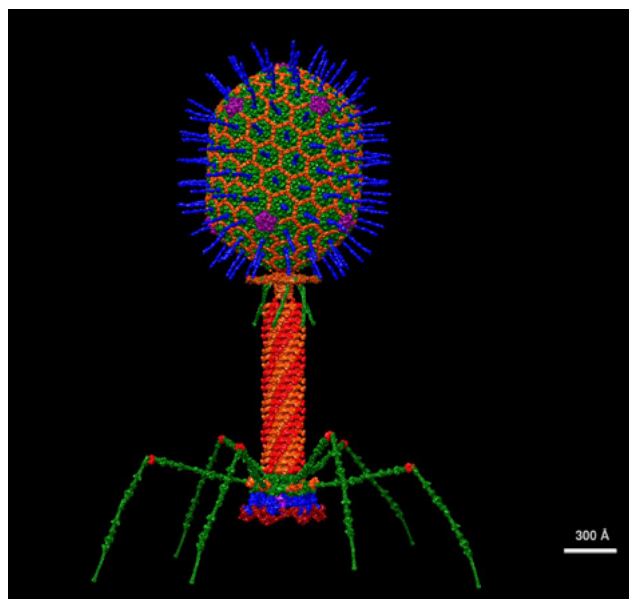


Figure 8. Structural model of the T4 phage based on protein structure data

Image: Victor Padilla-Sanchez/Wikipedia, CC BY-SA 4.0

Questions to aid the discussion:

- Is there anything about the plates that becomes more noticeable as the pH becomes more acidic?
- Knowing what you now know about bacteriophages, what do you believe this decrease in plaques is due to?
- Can you predict what would happen if we were to add an alkali, that is, make the pH more basic?

Conclusion

Phages are fascinating organisms and quite engaging, with their weird spaceship structures and the fact that they are viruses that attack bacteria.

The fact that loss of viability leads to the clear and countable visual readout of plaques makes them a good system for demonstrating protein denaturation. Students may also be interested to know that the same plaque-counting approach is used by phage researchers when evaluating different phage mutations or application.

In addition to all the discussion possibilities mentioned above, a debate or discussion can be held on whether phages can be used as a treatment against antibiotic-resistant bacteria. This is an obvious question on learning that phages kill bacteria, and if students ask this question, they may be interested to know that phages are indeed being investigated as medical treatments for antibiotic-resistant infections.^[1,2] The potential and challenges of this approach are something students could research independently and present to the class. <<

References

- [1] Strathdee SA et al. (2023) [Phage therapy: from biological mechanisms to future directions](#). *Cell* **186**: 17–31. doi: 10.1016/j.cell.2022.11.017
- [2] Brives C, Pourraz J (2020) [Phage therapy as a potential solution in the fight against AMR: obstacles and possible futures](#). *Palgrave Communication* **6**. doi: 10.1057/s41599-020-0478-4

Resources

- Watch a video on [bacteriophages and their potential for use in medicine](#).

- Watch a beautiful animation of [phage structure and infection of a bacterial cell](#).
- This second animation explains [how phage particles are assembled](#).
- Watch the second section (0:17 to 0:57) of this slightly older video for an excellent [animation of phage structure and infection](#).
- Understand how phage plaques are formed: Abedon ST (2021) Detection of bacteriophages: phage plaques. In Harper DR et al. (eds) *Bacteriophages* pp 507–538. Springer. ISBN: 978-3-319-41985-5
- Find similar experiments that can be used with students: Richter M, Fraser D (1959) [The use of bacteriophages in high school biology](#). *The American Biology Teacher* **21**: 282–287. doi: 10.2307/4439165
- Discover how bacteria from ants' feet could help us reduce pesticide use: Jensen IC (2024) [Footprints in the agar: growing bacteria from ants' feet to combat plant diseases](#). *Science in School* **67**.
- Use lactase to explore the biochemistry of sugars and the properties of enzymes: Rautenstrauch H, Ruppertsberg K, Thomsen S (2024) [Explore enzymes and the science of lactose intolerance using lactase tablets](#). *Science in School* **66**.
- Explore viscoelasticity by making slime: Ospina V, Ospina C (2024) [Beyond solids and liquids: the science of slime](#). *Science in School* **67**.
- Learn about the biochemical aspects of honey through a series of simple experiments: Scheuber T (2023). [To bee or not to bee: the biology of bees and the biochemistry of honey](#). *Science in School* **62**.
- Take a virtual tour of the world of molecular biology: Reiriz Martinez E, Hall SL (2024) [Explore the world of molecular biology without leaving the classroom](#). *Science in School* **68**.
- Explore the structure of biomolecules and create artworks that illustrate their beauty: Gupta D, Armstrong D (2021) [Introducing students to the beauty of biomolecules](#). *Science in School* **53**.
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- Discover how artificial intelligence is helping to predict protein folding: Heber S (2021) [From gaming to cutting-edge biology: AI and the protein folding problem](#). *Science in School* **52**.
- Discover how egg cells 'zips up' after fertilization to stop more sperm from entering: Capellas Espuny M (2024) [How does the body avoid multiple sperm fertilizing an egg?](#). *Science in School* **69**.
- Explore the fascinating world of noncoding RNAs: Koskova Z, Hernandez M (2023) [Not just a blueprint for proteins: the importance of non-coding RNAs](#). *Science in School* **65**.
- Read about how modern vaccines work: Paréj K (2021) [Vaccines in the spotlight](#). *Science in School* **53**.

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