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**Characterising a novel HNH-domain
containing bacterial immunity system from
*Escherichia coli***

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Characterising a novel HNH-domain containing bacterial immunity system from *Escherichia coli*.

Abstract:

Bacteria are constantly in danger of being attacked by bacterial viruses - bacteriophages. To combat the threat, bacteria have developed a wide array of intricate and diverse immune systems. Phage therapy's growing popularity has led to increased interest in bacterial immunity. My thesis investigates one such potential immunity system from *Escherichia coli*, discovered by our colleagues at Lund University, named Bogomol. It is predicted to be comprised of an HNH-containing endonuclease domain and a C-terminal domain of unknown function. I validated Bogomol as an immunity system acting against certain phages of *Straboviridae*. This was done by performing efficiency of plating assays and liquid culture experiments against the BASEL phage library. As an attempt to further investigate the triggers of Bogomol, escape mutant experiments were also conducted.

Keywords:

Bacterial immunity systems, bacteriophages, bacteriophage immunity assays

CERCS: B230 Microbiology, bacteriology, virology, mycology

Institute name: Institute of Technology

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Uudse, HNH domeeni sisaldava, *Escherichia coli* immuunsussüsteemi iseloomustamine

Lühikokkuvõte:

Bakterid elavad pidevas ohus sattuda bakteriaalsete viiruste ehk bakteriofaagide rünnaku alla. Viirustega võitlemiseks on bakteritel välja kujunenud lai valik keerukaid ja mitmekesiseid immuunsüsteeme. Faagiteraapia populaarsuse kasvuga on hüppeliselt tõusnud ka huvi bakteriaalse immuunsuse uurimise vastu. Minu lõputöö keskendub uudse ennustatud *Escherichia coli* immuunsüsteemi, Bogomoli, uurimisele, mille avastasid meie kolleegid Lundi ülikoolist. Bogomol koosneb HNH endonukleaasi domeenist ja teadmata funktsiooniga C-terminaalsest domeenist. Oma töös näitan ma, et Bogomol on immuunsüsteem, mis kaitseb baktereid osade *Straboviridae* perekonna bakteriofaagide vastu. Selleks testisin ma kui tõhusalt BASELi faagiraamatukogu viirused tard- ja vedelsöötmel Bogomoli ekspresseerivat bakterikultuuri nakatavad. Lisaks üritasin ma eraldada faagide põgenemismutante, et uurida Bogomoli aktiveerimise mehhanisme.

Võtmesõnad:

Bakteriaalsed immuunsussüsteemid, bakteriofaagid, bakteriofaagide immuunsusanalüüsid

CERCS: B230 Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

Abi - Abortive infection

BASEL - BActeriophage SElection for your Laboratory

CRISPR-Cas - clustered regularly interspaced short palindromic repeats

Dpi - Dots per inch

dsDNA - Double-stranded DNA

HNH - Endonuclease domain named for characteristic histidine and asparagine residues

λ - Lambda

MOI - Multiplicity of infection

OD - Optical density

RM - Restriction-modification

RPM - Rotations per minute

SM (buffer) - Sodium chloride and magnesium sulphate containing buffer

INTRODUCTION

Bacteriophages are viruses that infect bacteria and pose a constant threat to bacterial populations. To defend against phage infections, bacteria have evolved a range of immunity systems. These immunity systems vary in mechanism and structure, but all serve the purpose of protecting bacterial cells from phage infection. With the rise of the antibiotic-resistance crisis, phage therapy has become a more compelling field of research than ever. Given all this, understanding how bacteria defend themselves against phages has become increasingly important.

This thesis focuses on validating and characterising one such system, Bogomol, which is a putative defence system discovered by our collaborators at Lund University. Bogomol is an unclassified defence system that is predicted to function through an HNH-containing nuclease domain, a motif known to be involved in nucleic acid cleavage. My aim was to test whether Bogomol is indeed involved in bacterial immunity and, if so, against which phage families it is active.

The research was conducted with Bogomol from *E. coli* Ec001. To investigate Bogomol's protective effects, I transformed *E. coli* BW25113 cells with a Bogomol-expressing plasmid and tested their resistance against the BASEL phage library using efficiency of plating assays and liquid culture infection experiments. Atop all this, an attempt to isolate escape mutants was made in order to explore potential triggers of the Bogomol defence system and further characterise its specific biochemical properties.

Using phenotypic assays and a diverse library of phages, this work aims to better understand Bogomol as a potential bacterial immunity system.

1 LITERATURE REVIEW

1.1 Overview of Bacteriophages

Bacteriophages, also commonly known as phages, are viruses that exclusively infect bacteria. They are obligatory parasites, which means that they depend on the host cell to thrive and reproduce. Phages were first discovered by Twort in 1915 (1) and d'Hérelle in 1917 (2) and were initially studied with the goal of being used as anti-bacterial agents. Later, they started to be implemented clinically, mainly in the former Soviet Union countries (3).

Double-stranded DNA (dsDNA) phages are the most abundant and well-studied type of bacteriophage. Most dsDNA phages share a common structure: an icosahedral capsid that has the viral genome inside, attached to a tail used for host recognition and DNA injection (Figure 1.). Many phages also possess tail fibres, which play an important role in the initial attachment to the bacterial surface and are often responsible for determining host specificity (4).

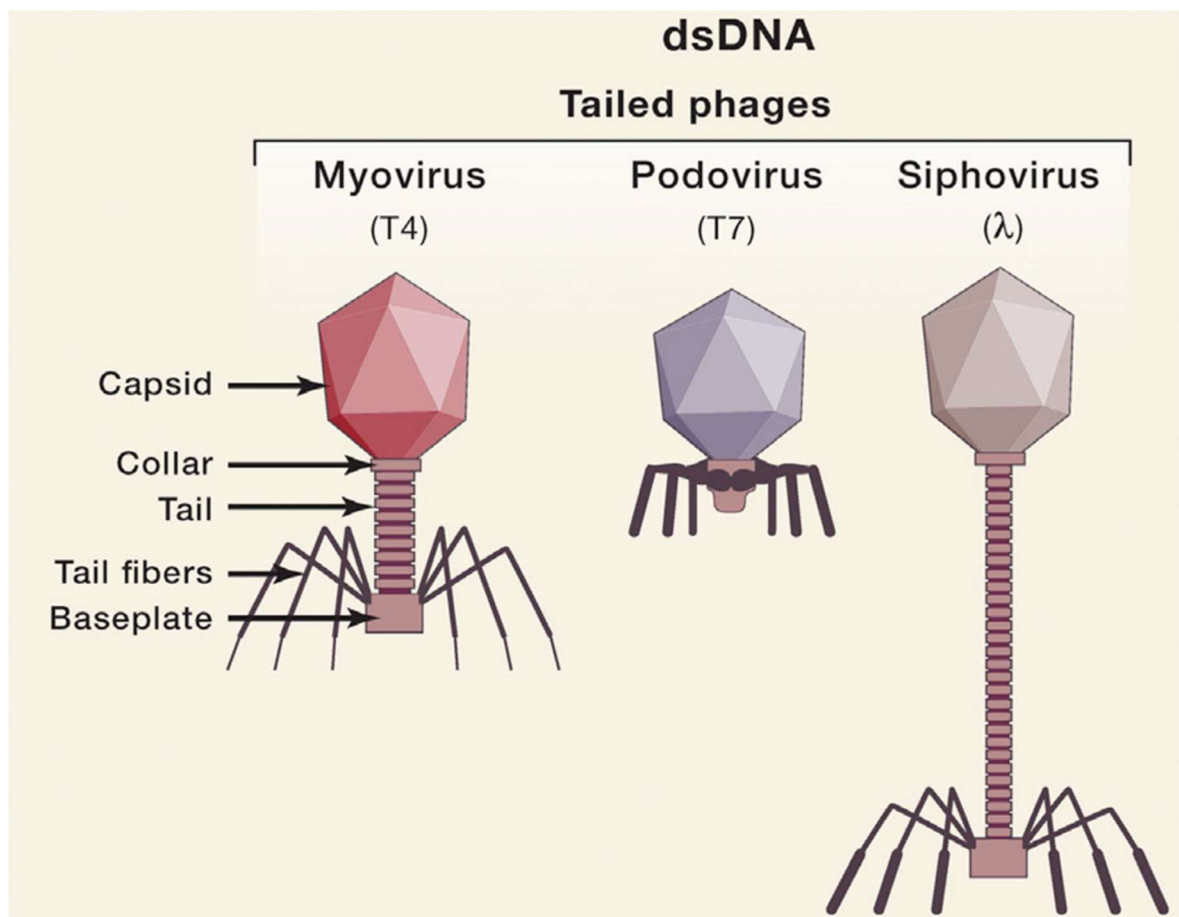


Figure 1. Morphology-based taxonomy of tailed dsDNA phages. Adapted from (11)

Based on tail morphology, these phages are generally divided into three main groups: *Myoviridae*, like T4, with long contractile tails; *Siphoviridae*, such as λ , which have long, flexible non-contractile tails; and *Podoviridae*, like T7, with short, stubby tails. These structural distinctions are closely linked to the different mechanisms by which each phage type infects its bacterial host (4).

Phages were discovered in the early 20th century and are now known to be the most abundant biological entities on the planet. Global phage diversity far exceeds that represented by cultured isolates, hence showing that they play a crucial role in our ecosystems (5).

1.1.1 Lytic Replication

During the lytic cycle, the phage attaches itself to the host cell, inserts its genome into the host's cytoplasm, which then, in turn, is given instructions to synthesise its proteins. After this, viral genomes and capsid proteins are rapidly made by the host cell, which assemble into multiple copies of the original phage that infected the host, as depicted in Figure 2. As the infection progresses, the host cell eventually dies and is lysed. This “bursting” action releases the new phages to infect neighbouring cells (6). Some phages are only lytic by nature, meaning they do not integrate into the host genome but always follow the lytic pathway (7).

1.1.2 Lysogenic Replication

The lysogenic cycle starts out similarly to the lytic cycle, with the phage attaching itself to the host cell. However, this time, the phage genome is integrated into the bacterial DNA (Figure 2.). This means that when the bacterial cell replicates, the viral genome can be passed on to daughter cells without killing them. Phage genomes integrated into bacterial chromosomes are known as prophages, and the host bacteria harbouring them are called lysogens. In response to environmental stress, prophages can switch to the lytic cycle, leading to the destruction of their bacterial host. (6)

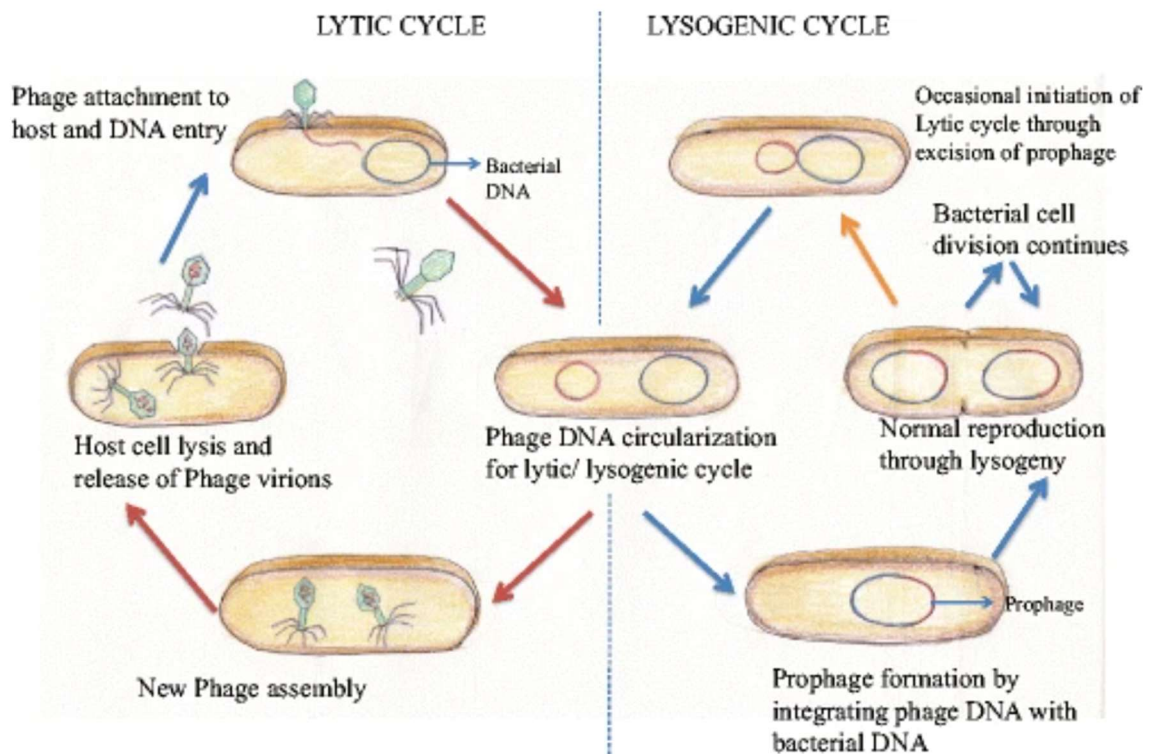


Figure 2. Schematic representation of the lytic and lysogenic cycles of phages. Adapted from (8).

1.2 Prophages

While often viewed as parasitic, prophages can provide several advantages to their bacterial hosts. They may carry genes that enhance bacterial fitness, such as toxin production, stress resistance, or immune defence systems. This symbiotic relationship can increase the host's competitiveness in complex microbial communities and plays a significant role in microbial evolution (9).

Many virulence genes found in prophages are arranged as independent genetic elements known as variable genetic regions. These typically have their own promoters and transcriptional terminators, allowing them to be expressed independently without interfering with nearby prophage genes that could be repressed during lysogeny. This genetic design not only ensures efficient expression during the lysogenic phase, but also facilitates horizontal gene transfer, since variable genetic regions can be easily exchanged between phages through recombination. They often have different G and C nucleotide content compared to the surrounding DNA, indicating a foreign source of this section of the genome. Overall, phages evolve by exchanging genetic elements with each other, which makes their genomes highly mosaic, which means it is like a mix of bits from different sources. Since many bacterial

strains carry multiple prophages, recombination between them can lead to large chromosomal rearrangements. If two prophages have similar DNA, they are more likely to recombine, which can cause large DNA rearrangements like deletions or inversions (10).

1.3 Biotechnological Relevance

The long-standing arms race between bacteria and phages has given us important molecular tools like restriction enzymes and CRISPR-Cas (11). With new bacterial immunity systems still being discovered, this area of research continues to be a goldmine for gene editing technologies. Some prominent examples are gene-expression systems based on the phage T7 RNA polymerase, the phi29 DNA polymerase, and the phage P1-derived Cre-Lox system that is used for site-specific recombination (11). The rapid expansion of known phage genomes has uncovered a surprising number of metabolic genes that can interact with important bacterial processes. These genes aren't just interesting from an evolutionary perspective, as they also hold potential for use in synthetic biology. For example, synthetic carbon fixation cycles are a major focus in biotechnology, and it's already known that phages can significantly influence carbon cycling in marine photosynthetic bacteria (11, 12). Some lysogenic phages have been found to carry intricate molecular decision-making systems, which could be repurposed for biological computation. In fact, phage recombinases have already been used to build logic circuits in cells (13).

1.4 Phage Therapy

Phage therapy, which involves using lytic phages to treat bacterial infections, has the potential to be a promising alternative to antibiotics, especially in the current era of rising antibiotic resistance. Unlike broad-spectrum antibiotics, phages are highly specific to their bacterial hosts, meaning they can target and eliminate harmful bacteria without disturbing the surrounding microbiota. This specificity, however, has both pros and cons, as effective treatment requires identifying or isolating phages that match the exact bacterial strain causing the infection. Recent clinical successes and personalised phage treatments have shown encouraging results, even in cases where all conventional treatments have failed. For example, certain phage therapy trials were successful at eradicating a uropathogenic strain of *Escherichia coli* in a patient, according to a study done in Russia (15). At the same time, challenges remain, particularly around regulatory approval, large-scale production, and understanding how phage-host interactions may evolve during therapy. Research is also increasingly focused on engineering phages or using them together with antibiotics to improve therapeutic outcomes and minimise the development of potential resistance (16,17,18,19).

1.5 Bacterial Immunity Systems

Bacteria are in a constant evolutionary arms race with bacteriophages. To survive in such a hostile environment under the predation of phages, bacteria have evolved a wide array of defence mechanisms to detect, neutralise, and prevent repeated phage infection. Although scientists have studied prophage-encoded antiphage defence systems for a long time, our more refined knowledge of the complexities of these systems is fairly recent. Immunity systems across all phage types are divided into three groups based on their action mechanisms: superinfection exclusion systems that block infection by preventing phage adsorption or genome entry, direct immune systems that eliminate phages without killing the infected cells, and abortive infection (Abi) systems, which block phage infection, but result in death of the infected cell (20).

1.5.1 Defence Islands

Recent studies have revealed that many of these defence genes are not scattered randomly but are instead clustered within genomic regions known as “defence islands,” which are gene operons for antiphage activity (21, 22). This discovery has increased our understanding of the bacterial pan-immune system, which is an understanding that although a single bacterium is unable to encode all anti-phage systems because of the high fitness cost, horizontal gene transfer allows mobile genetic elements to share these systems within a given bacterial population (20). Many of the mobile genetic elements found in bacteria are prophages. Prophages encode the genes essential for replication and viral assembly, as well as additional genes that provide their hosts with selective advantages (23). These defence systems give prophages a layered resistance strategy, which also protects the host cell and boosts the chances of the whole bacterial population surviving a phage attack (20).

1.5.2 Preventing phage adsorption:

Phage infection begins when the virus attaches to a specific receptor on the bacterial cell surface, also known as adsorption. To counter this, some prophages encode adsorption inhibitors, which serve two main purposes. Firstly, they protect the bacteria from being re-infected by other phages that target the same surface receptor. Secondly, they help ensure that newly produced phage particles aren't wasted on infecting cells that already contain a prophage by blocking access to the receptor used for entry (20).

Some prophages in *Pseudomonas* have developed ways to block phage infection by interfering with common surface receptors. For example, the prophage D3112 carries a gene for Tip

(twitching inhibitory protein), which binds to PilB - the ATPase needed to build type IV pili. PilB is needed for the pilus to form on the surface, which stops phages that rely on it from getting in, so without it, the pilus doesn't assemble, and those phages can't attach or inject their DNA, essentially blocking the infection before it even starts (24).

1.5.3 Blocking phage genome injection

After adsorption, the next critical step for successful infection is the delivery of the phage genome across the bacterial envelope via injection. This stage presents another opportunity for prophage-encoded defences to act and help prevent infection. Some of these systems involve membrane proteins that either block genome entry directly or interfere with components involved in the process. For instance, the mycobacteriophage Charlie encodes a protein that helps block some transmembrane proteins, hence preventing phage DNA from crossing into the cytoplasm (25). These systems, which target phage entry mechanisms rather than adsorption, tend to be specific to a narrower range of phages but provide a strong advantage, because, unlike surface blockers, they neutralise phage particles and stop the spread of infection altogether whilst maintaining the host bacteria alive (20).

1.5.4 Preventing phage replication after genome entry

Once inside the cytoplasm, the phage genome becomes vulnerable to intracellular defence systems aimed at blocking replication and stopping phage progeny. These mechanisms generally fall into two categories: those that act quickly to inhibit early stages of infection, and those that trigger cell death through Abi. The first can rescue the infected bacterium cell by stopping the phage in its tracks, while the latter sacrifices the infected host for the benefit of the surrounding community (16).

1.5.5 Direct immune systems

CRISPR-Cas and restriction-modification systems (RM) are the two most well-known bacterial immunity systems. The CRISPR-Cas system functions like a genetic memory. Bacteria incorporate fragments of phage DNA into their genome, which are later transcribed into guide RNAs that help Cas proteins recognise and cleave matching foreign DNA during future infections (26). RM systems, on the other hand, rely on restriction enzymes that cut foreign (unmethylated) DNA at specific sites, while bacterial DNA is protected by methylation (18) (25). Alongside these, phage repressor proteins can also play a role in immunity by binding to and suppressing regions of the prophage genome to keep it in a lysogenic state.

These repressors can also provide immunity by suppressing incoming phage genomes that enter the cytoplasm, blocking infection and ensuring the survival of the bacteria (27).

1.5.6 Abortive infection

Abi systems work by sacrificing the infected cell to stop the phage from spreading in the population. In these cases, the phage manages to enter the cell, but its life cycle is interrupted, and very few to no new viruses are made. Even though the infected cell dies, this helps protect the rest of the bacterial population from a phage outbreak (16).

The first known Abi system was RexAB, found in phage λ (lambda). When a λ -carrying cell is infected by phage T4, RexAB detects the T4 replication machinery and triggers cell death. Similar systems have now been found in *Mycobacterium* and *Gordonia*, where the protein RexA senses the infection and activates another protein RexB, which forms a channel in the cell. This causes the cell to lose its membrane potential and die (28).

Toxin-antitoxin systems are another form of Abi defence. These involve a stable toxin, which can harm or kill the cell, and a less stable antitoxin that keeps it under control. When a phage infects the cell, this balance is disrupted, and the toxin is released to fight the infection. For example, the HigBAC and CmdTAC systems in *E. coli* offer protection against phages by interfering with translation. In both cases, the toxin-antitoxin systems also encode for a chaperone, which stabilizes the antitoxin. When λ phage infects the cell, HigC starts to interact with newly synthesized tail-tube protein, destabilizing the antitoxin and leading to active HigB toxin. HigB halts the cell growth by cleaving RNA. In a separate system, the CmdT toxin modifies mRNA through ADP-ribosylation, effectively halting protein synthesis. Even though these systems limit phage replication, the cellular damage often results in the death of the host cell (29).

1.6. Bogomol:

Our partners at Lund University, who are part of Gemma Atkinson's group, screened Enterobacteria prophages for variable genetic regions and predicted potential novel immunity systems (unpublished data). One promising hit was the HNH endonuclease domain containing a previously undescribed protein family, which was code-named Bogomol (translating to Praying Mantis in Russian), due to its AlphaFold predicted structure containing long whiskers resembling Praying Mantis raptorial forelegs (Figure 3.).

The HNH is a widespread metal-dependent endonuclease domain, found in site-specific homing endonucleases, colicins, restrictases, CRISPR-Cas9 and DNA repair proteins (30).

Structurally, it folds into a $\beta\beta$ - α -metal motif, where a divalent metal ion is located in the active centre. The HNH active site consists of two highly conserved amino acids, histidine and asparagine, and a variable histidine can sometimes be replaced with asparagine (31). The second domain of Bogomol is of unknown function. My project was to test whether Bogomol indeed acts in phage defence by testing its phage defence capabilities in *E. coli* against the BASEL phage library.

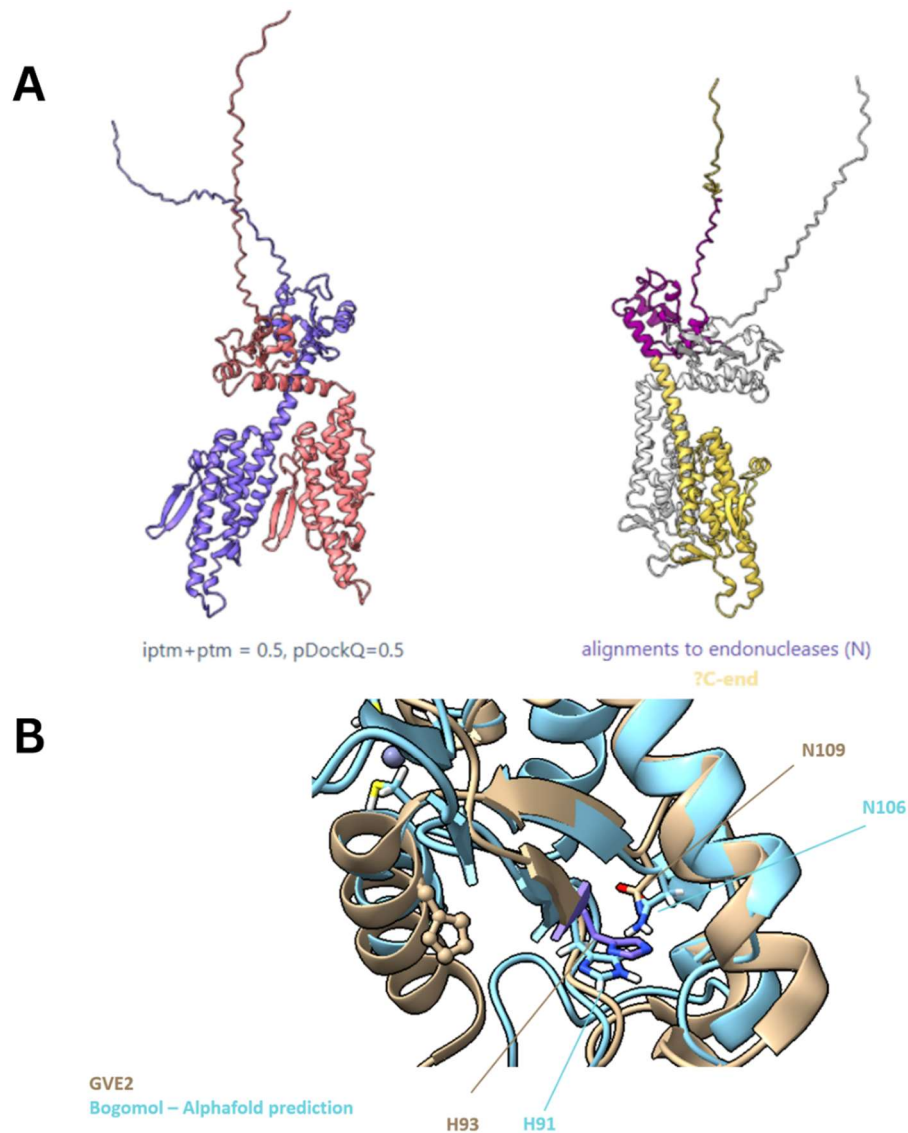


Figure 3. Structural prediction of Bogomol. **A)** AlphaFold predicted dimers of Bogomol from *E. coli* Ec001 visualised with ChimeraX version 1.8. The left panel visualises two dimers in red and violet, the right panel colours the predicted HNH endonuclease domain (purple) and unknown domain (yellow). Adapted from Artyom Egorov, unpublished. **B)** HNH domain active centre of Bogomol (blue) aligned with HNH domain of GVE2 bacteriophage (brown) (31). Shown are the highly conserved histidine and asparagine residues. Adapted from Toomas Mets, unpublished.

THE AIMS OF THE THESIS

The aim of my thesis was to test whether Bogomol acts as an immunity system in *E. coli*.
More specifically to:

- Test whether Bogomol can protect the standard *E. coli* lab strain BW25113 from phages in the BASEL phage library with a plaque assay.
- Further test the hits from the plaque assay in the liquid culture assay.
- Following the identification of potential immunity, attempt to isolate escape mutants.

2 EXPERIMENTAL PART

2.1.1 Materials and Methods

In this work, I used *E. coli* BW25113 as a standard bacterial strain (32). I transformed pBR-Bogomol and VHp1423 plasmids (unpublished) into *E. coli* BW25113 chemically competent cells. VHp1423 is a version of the pBR322 plasmid (33) that has its tetracycline resistance gene taken out, but the promoter is still there (Figure 4.). We used LB media (BD DIFCO™) supplemented with carbenicillin 100 µl/ml (Cb) (Fisher Bioreagents) for plasmid selection. For phage experiments, I used LB media enriched with 20 mM MgSO₄ and 5 mM CaCl₂ salts and 0.2% glucose. All assays were done against the BASEL (BActeriophage SElection for your Laboratory) phage library and a set of common laboratory phages: BASEL1-69, λ, P1, P2, N4, T2, T4, T5, T6 and T7 (34).

2.1.2 pBR322

pBR322 is a synthetic plasmid vector created for use in *E. coli*. Being only about 4361 base pairs long, its short length and presence of multiple unique restriction sites make it highly versatile for cloning experiments. pBR322 also possesses two antibiotic resistance genes: amp^R, which gives ampicillin resistance and tet^R, which provides it with tetracycline resistance. Moreover, its origin of replication (ori) is from ColE1, allowing it to replicate in *E. coli*. The availability of its full nucleotide sequence and dual antibiotic resistance markers gives researchers huge advantages for selection and screening (33). In our phage experiments, the tetracycline resistance gene in the pBR322 vector is replaced with a hypothetical immunity system. P_{tet} drives the expression of our immunity systems, and transcription is stopped by the added rrnB terminator (Figure 4.).

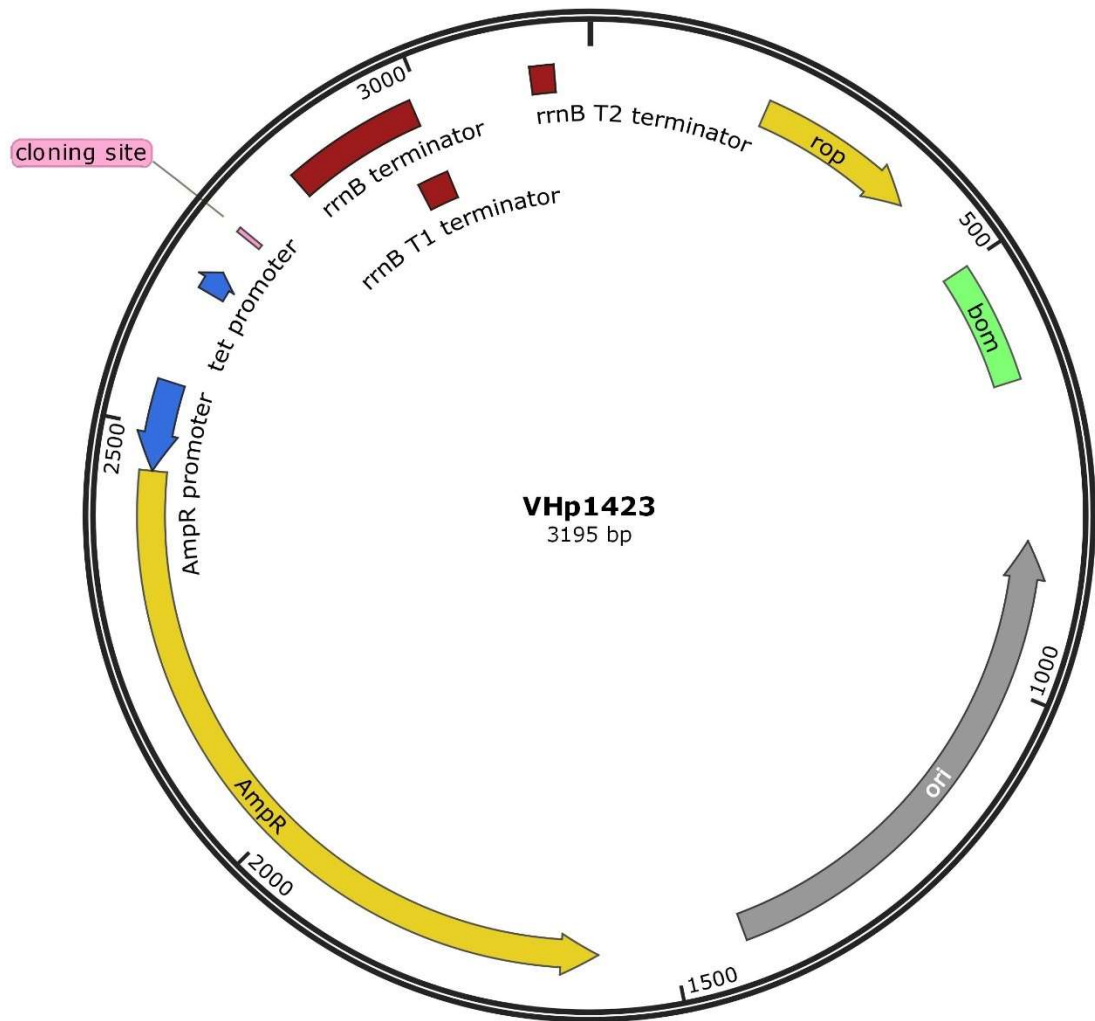


Figure 4. Map of VHp1423 plasmid. VHp1423 is a derivative of the pBR322 plasmid, where the tetracycline resistance gene has been removed, but the promoter is left intact. The vector is used to constitutively express genes using P_{tet} promoter. rrnB terminator from pBAD33 has been added after the cloning site to stop the transcription. The plasmid carries an Ampicillin resistance gene (AmpR) driven by its own promoter, allowing for antibiotic selection.

2.2 Bacterial Transformation

I took competent *E. coli* BW25113 cells from a -80°C freezer and put them on the ice to melt for 10 minutes. Next, I added 1 µl (50 ng/µl) of pBR-Bogomol or pBR322 plasmid to the 50 µl of competent cells in a 1.5 ml tube and lightly flicked the tube to ensure proper but gentle mixing. After incubating the mixture of plasmids and competent cells on ice for 20-30 minutes, I performed a heat shock for 1 minute at 42°C on a heat block (ThermoStat C,

Eppendorf). Immediately after the heat shock, I put the mixture on ice for recovery and incubated it for 5 minutes. I added 600µl of LB medium supplemented with 0.2% glucose to the tubes and put the transformants into a 37°C shaker, Thermomixer compact, at 1000 RPM for 45 to 60 minutes to recover them.

After recovery, I placed 100µl of the grown bacterial cultures onto LB agar plates supplemented with 100 µl/ml of Cb. The bacteria were spread on the plate by quickly rubbing it on the agar using a properly sterilised glass spatula on a rotating platform. Finally, the plates were placed in a 37°C incubator overnight.

2.3 Efficiency of Plating Assay

2.3.1 Top Agar Preparation

I transformed appropriate plasmids into *E. coli* BW25113 cells as described above. Following that, I inoculated a single colony of transformant into 3 ml of LB broth containing 100 µg/ml CB and grew the culture at 37°C while shaking overnight. I then diluted the overnight culture into around 43°C top agar to an optical density at 600 nm, or OD₆₀₀, of 0.075. The top agar was 0.5% LB agar supplemented with 5mM CaCl₂ and 20mM MgSO₄. I then poured 6 ml of this prepared top agar mixed with bacterial culture on LB Omni Tray plates with ready agar and left them to dry slowly without a lid, whilst under the flame.

2.3.2 Phage Dilution Preparation:

To prepare the phage dilutions, I used sterile flat-bottomed 96-well plates (Anicrin). I filled each well except the first row with 90µl of SM buffer, as the first row received 180µl of SM buffer. SM buffer is a buffer used for diluting and storing bacteriophages. SM buffer contains: 200 mM NaCl₂, 10 mM MgSO₄, 50 mM Tris-HCl, pH 7.5 (35). Next, using filtered pipette tips, I added 20µl of phage working stock to the first row, making sure each column has a different type of phage. All phages were obtained from the BASEL phage library (34). The phage working stocks are all in concentrations of 10⁹ to 10¹¹ plaque-forming units per millilitre (PFU/ml). Using a multichannel pipette, I performed a series of 10x serial dilutions of phages by transferring 10 µl of culture to the next row of wells. Afterwards, using a multichannel pipette again, I spotted 2.5µl of the phage dilutions on the top agar plate. Upon completion, I dried the plates by the flame.

The plates were then incubated at 37°C overnight. Images of the plates at 300 dpi resolution were taken after 6 and 24 hours using an Epson Expression 1680 Pro scanner.

2.4 Preparing Phage Stock

To replenish our supply of phages for the upcoming liquid culture experiment, I began by preparing fresh working stocks from nearly finished tubes. First, I inoculated 3 ml of LB medium with a single colony of *E. coli* BW25113 and incubated it overnight at 37°C while shaking at 220 RPM.

Then, I prepared a soft agar overlay by melting LB top agar (0.5% agar) and supplementing it with 5 mM CaCl₂ and 20 mM MgSO₄. Once cooled, I mixed the soft agar with 100 µl of the overnight bacterial culture and then quickly smeared phage stocks into the top agar with a sterile loop. The plate was left to solidify under the flame and then incubated overnight at 37°C.

The next morning, the plates contained phage lysis zones. I then scraped the surface with a sterile 1 ml pipette tip to pick up the phage particles containing gloop. The resulting phage suspension was collected into a 2 ml tube and centrifuged at 15000 RPM for 10 minutes to pellet debris. The supernatant was then collected and stored in fresh 1.5 ml tubes at 4°C. Phage stock concentrations were determined using serial dilutions, using BW25113 as a host.

2.5 Liquid Culture Assay

For this set of experiments, I first prepared overnight cultures of BW25113 containing pBR322 or pBR-Bogomol by inoculating a colony of each into 3 ml of LB media with 100 µl/ml of Cb. The next day, I diluted the overnight culture into LB media to OD₆₀₀ = 0.075. The LB media was supplemented with CaCl₂ 5mM and MgSO₄ 20mM. For this part of the experiment, 100 µl of bacterial culture was dispensed into each well of a flat-bottomed 96-well plate (Anicrin), except for row A, which contained only LB medium. Row B received 10 µl of SM buffer, while the remaining wells received 10 µl of various phage dilutions. Half of the plate was filled with cultures transformed with pBR322, and the other half with Bogomol-transformed cultures. The plates were run on a SPECTROstar Nano BMG LAB-TECH plate reader for 6 hours, measuring OD₆₀₀ every 15 minutes at 37°C while shaking at 400 RPM.

2.6. Escape Mutant Assay

For this experiment, I began by preparing overnight cultures of bacteria transformed with the pBR-Bogomol plasmid and incubating them at 37°C. The following day, I added 20 µl of serially diluted phage stocks to 100 µl of the overnight culture. Each mixture was then combined with 3 ml of melted top agar and poured onto solid LB agar plates. After allowing the top agar to solidify for 20–30 minutes, the plates were incubated overnight at 37°C. The next day, I examined the plates for normal-sized plaques.

2.7. Reproducibility

The initial efficiency of the plating assay to screen for affected phages was performed once for the whole phage collection. The bacteriophage-host interactions identified as potential hits were later re-tested independently at least two extra times to confirm consistency. Liquid culture experiments were conducted in biological triplicate, with all replicates grown and measured on the same flat-bottomed 96-well plate to minimise the technical variability.

2.8. Additional Materials

GraphPad Prism was used for the analysis and visualisation of growth curves, while SnapGene was used to visualise plasmid maps. I used Canva to edit the LB agar plate images. ChimeraX was used to create the predicted 3D images of Bogomol. In addition to digital software, AI tools such as ChatGPT and Perplexity AI were used as a source of writing inspiration, a grammar check, an aid to help understand complex scientific concepts better and to help find relevant literature related to my topic. Above all, I frequently use Grammarly to ensure the grammatical integrity of my writing.

3 RESULTS AND DISCUSSION

3.1 Bogomol protects *E. coli* against select *Straboviridae*

Our colleagues at Lund University in Gemma Atkinson's group predicted a new putative HNH domain-containing immunity system, named Bogomol, during a defence system screen in enterobacteria. To test whether it really defends enterobacteria against phages, my colleagues cloned Bogomol from *E. coli* Eco001 into pBR322 vector with its native Shinde-Dalgarno sequence under the P_{tet} replacing the tetracycline resistance gene. In this setup immunity system is constitutively expressed. Fresh transformants of *E. coli* BW25113 cells containing either empty vector or pBR-Bogomol were used to start overnight cultures. The next day, top agar plates were poured with these overnight cultures and tested in parallel against the BASEL *E. coli* dsDNA phage library and 9 common laboratory phages (34) as presented in Figure 5. Scanned images of the plates were taken at 6h and 24h time points after incubation at 37°C. In this work, I only present the results for the 6-hour time point, as the results stayed the same between it and the 24-hour time points.

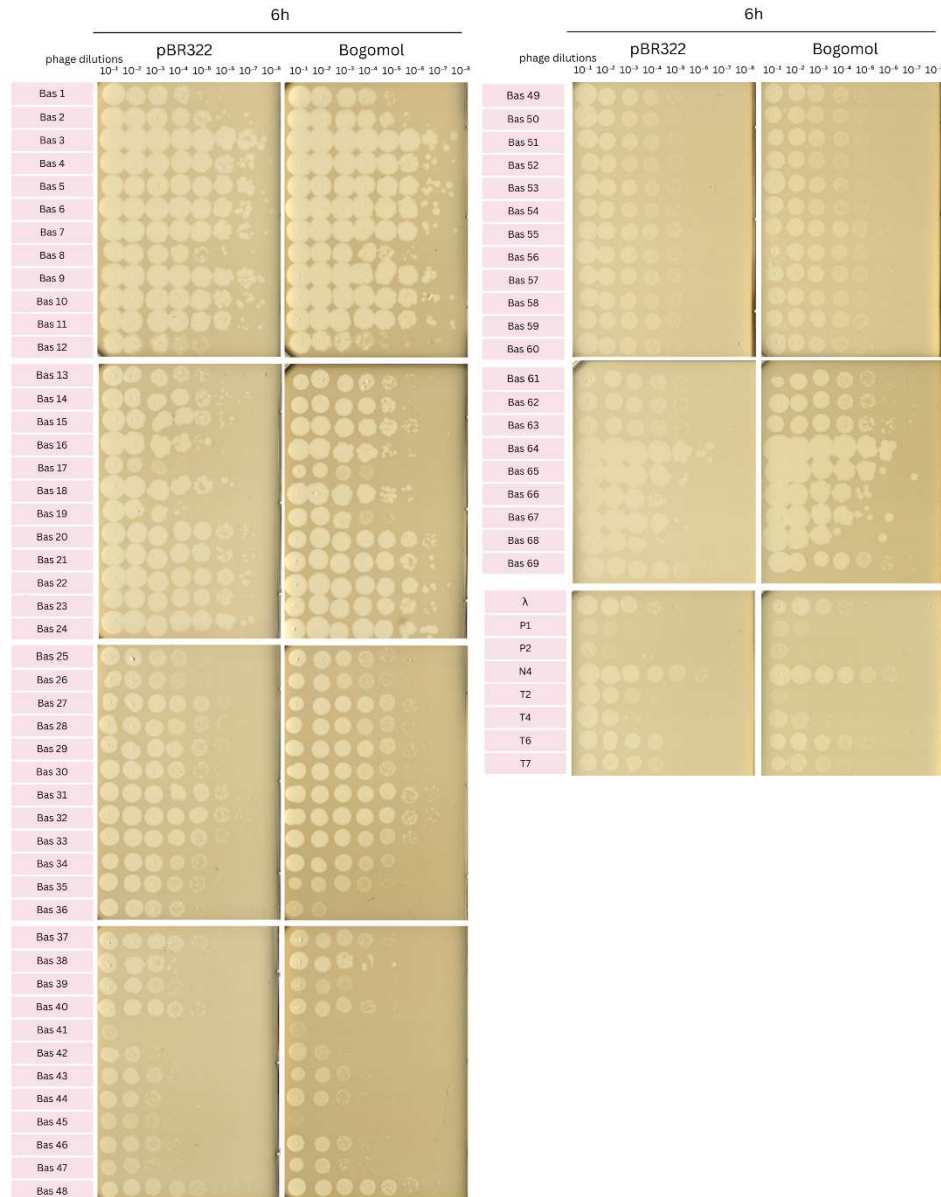


Figure 5. Efficiency of plating assay with Bogomol expressing *E. coli* against BASEL phage library. Overnight cultures of *E. coli* BW25113 transformed with pBR or pBR-Bogomol plasmids were made into a top agar lawn. The top agar is comprised of 0.5% LB agar medium supplemented with 5 mM CaCl_2 and 20 mM MgSO_4 . Serial 10-fold dilutions of each phage (10^{-1} to 10^{-8}) were spotted on bacterial lawns. Images were taken after 6 hours of incubation at 37°C .

The efficiency of the plating assay (Figure 5.) showed that Bogomol offers protection against T2, Bas36 and Bas45 phages. All three of these phages belong to the *Straboviridae* phage family. I further re-tested these hits, as demonstrated in Figure 6.

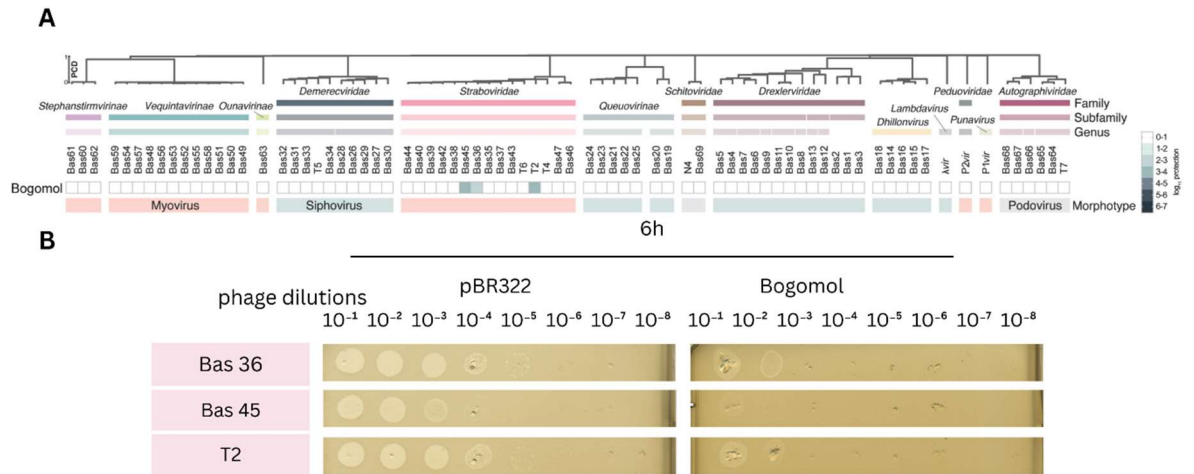


Figure 6. Phage protection profile of Bogomol. **A)** Heatmap representing the phage efficiency assay against Bogomol. The top half depicts the phylogenetic tree of screened phages. The grid below colourimetrically illustrates the phages against which Bogomol demonstrated immunity against as various shades of green. The colour scale on the right shows bacterial protection expressed as the logarithmic (log₁₀) reduction in plaque formation. The lighter shades indicate weaker protection, while darker shades reflect stronger protection against bacteriophages. **B)** Phage spot assay of Bas36, Bas45, and T2 against *E. coli* BW25113 transformed with pBR322 or Bogomol-containing plasmids after 6 hours of incubation at 37°C . Serial 10-fold dilutions of each phage (10⁻¹ to 10⁻⁸) were spotted on bacterial lawns.

As we can see in Figure 6., there is a clear reduction in plaque-forming ability of Bas36, Bas45 and T2 phages in the presence of the Bogomol plasmid, suggesting Bogomol indeed acts as an immunity system. For Bas36, plaque formation ceased at the 10⁻⁴ dilution in Bogomol expressing bacteria, whereas the control showed plaques up to 10⁻⁶, indicating a 100-fold reduction in detectable PFU (plaque forming units). In the case of Bas45, Bogomol completely inhibited plaque formation already at the 10⁻² dilution, while the control continued showing plaques up to 10⁻⁵, suggesting at least a 1000-fold reduction in phage propagation. Similarly, T2 showed plaques in the 10⁻³ dilution in Bogomol, compared to up to 10⁻⁶ in the control, again pointing to a three orders of magnitude decrease in phage virulence. Together, these observations demonstrate that the presence of the Bogomol plasmid equips the bacteria with protection against some phages of the *Straboviridae* family.

3.2 Liquid Culture Assay with Bas36, Bas45 and T2

Next, my aim was to assess the effectiveness of Bogomol against the phages that showed hits in the plaque assays, using liquid culture experiments. Bacteria were grown in a plate reader with a starting OD600 of 0.075 in the presence of phages with varying multiplicities of infection (MOI). The higher the OD600 measurement, the less phage-induced lysis has occurred, indicating potential bacterial immunity. In the control strains, increasing MOIs of all three phages caused progressively stronger inhibition of bacterial growth, with complete suppression seen at MOI 10 (Figure 7.). In contrast, Bogomol-expressing strains showed markedly improved growth across all MOIs for Bas45, and at MOI 1 or below for Bas36 and T2, indicating immunity (Figure 7.). Notably, Bogomol has demonstrated almost complete protection against Bas45 and substantial protection against Bas36 and T2. This is seen as the higher OD600 values compared to the control culture, even at high MOIs. These results conform perfectly with the plaque assay data and support the role of the Bogomol plasmid equipping the bacteria with protection against select phages of the *Straboviridae* family.

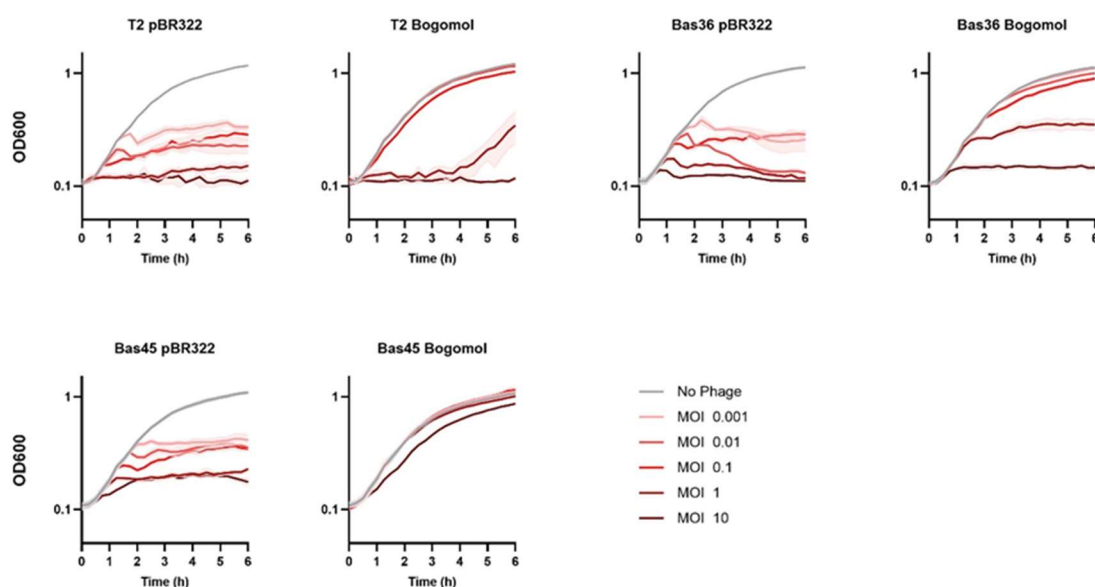


Figure 7. Liquid culture infection assay with T2, Bas36 and Bas45. Growth curves of *E. coli* BW25113 transformed with pBR322 or pBR-Bogomol in the presence of phages T2, Bas36, and Bas45 at varying MOI. Overnight cultures of BW25113 expressing either pBR or pBR-Bogomol plasmids were diluted to an OD600 of 0.075. 100 μ l of culture was infected with 10 μ l of various phage dilutions. Measurements were taken every 15 minutes over a 6-hour period at 37°C.

3.3 Attempts to Isolate Escape Mutants

In an attempt to get a better understanding of the mechanisms behind how Bogomol offers protection to *E. coli*, I proceeded to try isolating phage escape mutants. In theory, these experiments could help reveal the exact triggers for Bogomol activation, hence painting a more vivid picture of the specificities of the immunity system in question. More specifically, this can be done by infecting immunity system expressing phages with high concentrations of phages and screening top-agar lawns for plaques with wild-type morphology, followed by genetic sequencing and analysis. For example, using a similar approach, our group has previously shown that the HigBAC immunity system is triggered by the major tail protein of the λ bacteriophage (29). Despite our group's previous success, I failed to isolate escape mutants, even after multiple repeated attempts.

This opens up many speculations as to why this is so. It could be that Bogomol's triggering is more complex than interaction with a single phage protein or protein complex. It might be detecting some wider change in cell physiology, making single-point mutations insufficient for escape. Another possibility is that the potential escape mutations might severely compromise the phage's ability to replicate, making sufficient expression in *E. coli* impossible. Indubitably, there is also the chance that the experimental conditions may not have been optimal for escape mutant selection.

3.4 Conclusions

As demonstrated by the plate and liquid assays, pBR-Bogomol containing cells indeed display immunity against some of the tested bacteriophages from the *Straboviridae* family.

Based on the graphs of the liquid culture assay, we can see that the line graph follows a similar shape in Bas36 and T2. At lower MOIs the growth is almost completely rescued, while at higher MOIs there is no increase in OD600. This hints at the possibility that Bogomol has features of Abi mechanisms in Bas36 and T2. If the mechanism of Bogomol is abortive, and all bacteria are infected, they all halt in growth, and there will be no growth. However, the graph showing the results for Bas45 displays a pattern that does not align with the expected shape of Abi mechanisms, but more with restrictive systems. The bacterial culture continues to grow even at high MOIs, hinting that there is some other defence mechanism at play. Bas36 and T2 appeared to trigger a similar mode of immunity, whereas Bas 45 might have been neutralised through an alternative pathway. It must also be noted that Bogomol did not protect against all members of the *Straboviridae* family, suggesting

that its activity may depend on specific features of the phages, rather than broad classification. Another potential reason could be that all the other *Straboviridae* bacteriophages (excluding Bas36, Bas45 and T2) have innate anti-immunity systems within them, allowing for successful bypass of Bogomol.

To understand the exact mechanisms behind Bogomol, further experiments are required. For example, it is currently assumed that the HNH domain in Bogomol acts as a nuclease, however, current evidence is not strong enough to reach such a conclusion, so DNA and RNA cleavage analysis is necessary. Pulldown experiments would be a way to investigate what triggers the Bogomol mechanism. These experiments work by tagging Bogomol, making isolated purification a possibility, and hopefully co-purifying the interacting trigger with Bogomol. Mass spectrometry can then be done on the isolate, and further, more confident conclusions can be drawn.

Bogomol is a novel bacterial immunity system that contains two domains. Although we are well aware of its HNH-containing domain, it also contains a domain of unknown function. So far, it hasn't been characterised in any previous studies. Because of its uniqueness, making direct comparisons to existing literature is either extremely limited or not possible at all. This makes Bogomol an exciting addition to the growing literature of bacterial immunity. All in all, Bogomol is another important piece of knowledge which can help us get closer to understanding bacterial immunity system mechanisms.

SUMMARY

With the recent rise of antibiotic resistance, the importance of understanding phage-bacteria interactions has become more relevant than ever before. Phage therapy has emerged as a promising alternative to traditional antimicrobial treatments. Studying bacterial immunity systems not only supports the development of more effective phage therapies but also provides valuable tools for molecular editing in biotechnology. The aim of my thesis was to test whether a novel predicted immunity system from *E. coli*, Bogomol, protects bacteria from bacteriophages.

In this work, I show that Bogomol indeed acts as an immunity system and protects the standard *E. coli* lab strain BW25113 against certain *Straboviridae* phages. I performed efficiency of plating assays against phages from the BASEL phage collection and a set of common laboratory phages, which showed that Bogomol-expressing cells have protection against a specific set of phages from the *Straboviridae* family: Bas36, Bas45 and T2. These hits were then tested in a liquid culture infection assay. The liquid culture data aligned with the plaque assay results, showing generally consistent protection patterns.

Bogomol acted with T2 and Bas36 phages as an abortive defence system, based on the complete inhibition of bacterial growth at high MOIs. However, Bogomol acted differently with Bas45. The bacterial growth showed little to no inhibition throughout all MOIs, suggesting that the bacteria were left intact. This demonstrates properties more characteristic of restrictive immunity systems, which leave the host untouched. Further research is needed in order to draw conclusions on the exact nature and working mechanisms of Bogomol.

Finally, an attempt to isolate escape mutants from the sensitive phages was made in order to better understand the triggers of the Bogomol immunity system. Unfortunately, no escape mutants were recovered. Overall, the results suggest that Bogomol is a novel bacterial immunity system, capable of providing defence against *Straboviridae* bacteriophages.

KOKKUVÕTE

Antibiootikumiresistentsuse laia leviku tõttu on faagi-bakteri interaktsioonide mõistmine olulisem kui kunagi varem. Faagiteraapia on paljulubav alternatiiv traditsioonilisele antibiootikumipõhisele ravile. Bakteriaalsete immuunsussüsteemide uurimine aitab välja töötada efektiivsemat faagiteraapiat ja aitab avastada ja arendada molekulaarse biotehnoloogia tööriistu. Minu lõputöö eesmärk oli testida, kas uudne ennustatud *E. coli* immuunsussüsteem Bogomol kaitseb baktereid viiruste eest.

Oma lõputöös näitan, et Bogomol käitub tõepoolest immuunsussüsteemina ja kaitseb standardset *E. coli* laboritüve BW25113 osade *Straboviridae* faagide eest. Plaatimise efektiivsuse katsed BASELi kollektsiooni ja standardsete laborifaagide näitasid, Bogomol pakub kaitset *Straboviridae* perekonna faagide Bas36, Bas45 ja T2 vastu. Bogomol kaitses antud kolme faagi eest ka vedelkultuuris teostatud nakatamiskatsetes.

T2 ja Bas36 faagide puhul käitus Bogomol vedelkultuurikatsetes kui abortatiivne immuunsüsteem- kõrge faagitiitri juures oli bakterite kasv täielikult pärsitud. Nakatamiskatsetes Bas45-ga suutsid aga Bogomoli ekspresseerivad rakud kasvada vedelsöötmes edukalt ka kõrge faagitiitri juures. See viitab pigem restriktiivsele immuunsusmehhanismile, kus nakatumine peatatakse ilma peremeesorganismi ohverdamata. Bogomoli toimemehhanismi kohta järeltutse tegemiseks on vajalikud täiendavad uuringud.

Antud töös üritasin ma ka eraldada faagide põgenemismutante, lootuses paremini mõista Bogomoli aktiveerimise mehhanisme. Kahjuks ei õnnestunud isoleerida ühtegi põgenemismutanti. Kokkuvõtvalt näitavad tulemused, et Bogomol on uudne bakteriaalne immuunsussüsteem, mis pakub kaitset *Straboviridae* bakteriofaagide vastu.

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