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Validating three anti-phage defense systems in *Pseudomonas putida*

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Abstract

Bacteriophages (phages) are viruses that infect bacteria, causing significant mortality. Due to this selective pressure, bacteria contain anti-phage defense systems, proteins which help to abrogate or reduce the effects of infection. As biotechnology employs genetic editing techniques to optimise microbial strains, understanding their genome is necessary to guide these decisions. Aside from efficient performance or production, anti-phage defense systems are an important consideration for minimising risks to bacterial survival. The soil bacterium *Pseudomonas putida* prominently figures in synthetic biology research due to its favourable characteristics. Despite this, the anti-phage defense systems of *P. putida*, and the biotechnological PaW85 strain, are not studied extensively due to the lack of isolated phages. In this study, we use the CEPEST *P. putida* phage collection to verify three predicted anti-phage defense systems, PD-T7-1, HerA/DUF4297 and RMII. Mutant strains lacking the respective systems were constructed and phage susceptibility assays were conducted to test the function of each system in anti-phage defense. Ultimately, the roles of PD-T7-1 and RMII could not be verified with the phages available, while HerA/DUF4297 was seen to have defensive function. Further, using a strain with an active site mutation in DUF4297 showed that the enzyme likely functions as a nuclease in the complex. In the future, the expansion of the CEPEST collection will enable to further test the potential anti-phage defense roles of PD-T7-1 and RMII. Additionally, the validation of defensive function initiates future research in characterising the activation mechanisms of HerA/DUF4297 in *P. putida*. Together, the findings expand the current body of knowledge of the anti-phage defense systems in *P. putida* and may improve future biotechnology applications.

Keywords

Anti-phage defense systems, bacteriophages, *Pseudomonas putida*, CEPEST, genomic engineering, PD-T7-1, RMII, HerA/DUF4297

CERCS: B230 Microbiology, bacteriology, virology, mycology

Institute name: Institute of Molecular and Cell Biology

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Kolme anti-faagikaitsesüsteemi valideerimine *Pseudomonas putida*'s

Lühikokkuvõte

Bakteriofaagid (faagid) on viirused, mis nakatavad baktereid ja põhjustavad suurt suremust. Selle selektiivse surve tõttu sisaldavad bakterid faagivastaseid kaitsesüsteeme – valke, mis aitavad nakkuse mõju vähendada või kõrvaldada. Kuna biotehnoloogias kasutatakse mikroobitüvede optimeerimiseks geenitehnoloogilisi võtteid, on genoomi mõistmine nende otsuste suunamiseks

hädavajalik. Lisaks efektiivsele toimivusele või tootlikkusele on faagivastased kaitsesüsteemid olulised bakterite ellujäämise riskide vähendamiseks. Mullabakter *Pseudomonas putida* on sünteetilise bioloogia uurimistöös tähtsal kohal tänu mitmetele headele omadustele. Vaatamata sellele ei ole *P. putida* ega biotehnoloogilise PaW85 tüve faagivastaseid kaitsesüsteeme ulatuslikult uuritud, kuna isoleeritud faagid puuduvad. Käesolevas uuringus kasutati CEPEST *P. putida* faagikogu, et kontrollida kolme ennustatud faagivastase kaitsesüsteemi – PD-T7-1, HerA/DUF4297 ja RMII – funktsioone. Konstrueeriti mutanttüved, millel puudusid vastavad süsteemid, ja viidi läbi faagitundlikkuse analüüsid, et testida iga süsteemi rolli faagivastases kaitses. Lõppkokkuvõttes ei suutnud me PD-T7-1 ja RMII rolle saadaolevate faagidega kinnitada, kuid HerA/DUF4297 näitas kaitsefunktsiooni. Lisaks näitas muteeritud aktiivsaidiga DUF4297 kasutamine, et see ensüüm tõenäoliselt toimib nukleaasina. Tulevikus võimaldab CEPEST-kogu laiendamine PD-T7-1 ja RMII võimalike faagivastaste kaitserollide edasist uurimist. Lisaks avab kaitsefunktsiooni valideerimine tee tulevastele uuringutele HerA/DUF4297 aktiveerimismehhanismide iseloomustamiseks *P. putida*'s. Kokkuvõttes laiendavad need tulemused *P. putida* faagivastaste kaitsesüsteemide praegust teaduslikku baasi ja võivad parandada tulevasi biotehnoloogilisi rakendusi.

Võtmesõnad:

Faagivastased kaitsemehhanismid, bakteriofaagid, *Pseudomonas putida*, CEPEST, genoomitehnoloogia, PD-T7-1, RMII, HerA/DUF4297

CERCS: B230 Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia

TABLE OF CONTENTS

TABLE OF CONTENTS **4**

TERMS, ABBREVIATIONS AND NOTATIONS **6**

INTRODUCTION **7**

1 LITERATURE REVIEW **9**

1.1 INTRODUCTION TO ANTI-PHAGE DEFENSE SYSTEMS 9
1.1.1 Bacteria and phage interactions 9
1.1.2 Bacterial anti-phage defense systems (classification) 9
1.1.3 Phage anti-defense systems and co-evolution 10
1.1.4 Spread of anti-phage defense systems in bacterial genomes 11
1.1.5 Phage defense locus prediction tools 11
1.2 THREE POTENTIAL ANTI-PHAGE DEFENSE SYSTEMS OF *PSEUDOMONAS PUTIDA* PaW85 12
1.2.1 HerA/DUF4297 12
1.2.2 PD-T7-1 13
1.2.3 Restriction-Modification Type II (RMII) 13
1.2.4 *Pseudomonas putida* and its applications 14
1.2.5 Use of genomic information in biotechnology 14
1.2.6 Relevance of anti-phage defense systems in applications 15
1.2.7 The CEPEST collection of *P. putida* phages 16

2 THE AIM OF THE THESIS **17**

3 EXPERIMENTAL PART **18**

3.1 MATERIALS AND METHODS 18
3.1.1 Bacterial strains, plasmids and media 18
3.1.2 Phages 19
3.1.3 DNA cloning 21
3.1.4 Engineering *Pseudomonas putida* mutant strains 23
3.1.5 Phage susceptibility assays 24
3.2 RESULTS 25
3.2.1 Phage susceptibility of strains lacking predicted defense loci 25
3.2.2 Phage susceptibility of ΔHerA/DUF4297 28
3.2.3 Verifying HerA/DUF4297 function 28
3.2.4 Increased protection of HerA/DUF4297 complementation strain 29
3.3 DISCUSSION 31
3.3.1 Unverified defensive role of the PD-T7-1 and RMII systems 31

3.3.2 Defensive role of HerA/DUF4297 32
3.3.3 Future directions 33

SUMMARY 34

REFERENCES 35

NON-EXCLUSIVE LICENCE TO REPRODUCE THE THESIS AND MAKE THE THESIS PUBLIC 41

TERMS, ABBREVIATIONS AND NOTATIONS

CTD – C-terminal domain

DUF – Domain of Unknown Function

ML – Machine Learning

NTD – N-terminal domain

PD-T7-1 – Phage Defense against T7-1

RMII – Restriction Modification type II

INTRODUCTION

Bacteria are widespread in diverse and even extreme, otherwise uninhabited conditions. Bacterial communities are shaped by abiotic and biotic factors, causing them to adapt to various selective pressures (Alsharif et al., 2020). Specifically, they are engaged in a constant battle with their viruses, bacteriophages (phages), which are an obligate parasite requiring a host for survival. By propagating infection and killing, phages significantly impact the regulation of diversity and physiology of bacterial populations in the colonised environments. Phages can also drive antibiotic resistance acquisition, enhance pathogenicity by providing virulence genes and facilitate survival in stressful conditions (Naureen et al., 2020). By regulating bacterial community composition, phages can cause considerable pressure, while also being able to provide advantageous traits for their host's survival.

Nevertheless, phages are a major cause of bacterial mortality, making it essential for bacteria to escape infection in the first place (Suttle, 2007). To ensure their survival, bacteria have evolved anti-phage defense systems, single genes or groups of genes providing resistance to phage infection. These mechanisms include sensing phage DNA or proteins to activate protective functions. Since phages overtake the host machinery to replicate, bacteria also indirectly sense infection by monitoring complexes with this function. The sensor modules then activate downstream effectors to interrupt the phage replication cycle (Georjon & Bernheim, 2023). Interactions with phages have led to extensive diversification of bacterial genomes, as defense genes have especially high rates of gain and loss compared to other functional classes (Puigbò et al., 2017). This may complicate the study of defense, requiring a dedicated approach to identify the systems within it.

As complete genome sequences have become available for many organisms, developments in bioinformatics are key in interpreting gene functions and evolutionary relationships. Machine learning (ML) tools are facilitating the study of the anti-phage defense field by making information more accessible and overcoming the limitations of experimental methods. This is significant for more rarely studied organisms, since the gap in knowledge can be filled by providing a starting point with predictions based on homology. Structural AlphaFold models are often relied on to search for known active sites and functional motifs, thus predicting defensive function in other organisms (Payne et al., 2022). The importance of antiviral systems in bacteria causes effective functions to be conserved and shared through horizontal gene transfer, nevertheless, each bacterial species encodes its own unique arsenal (Tesson et al., 2022).

Therefore, to expand the knowledge of species-specific anti-phage defense, we focus on the environmental model bacterium *Pseudomonas putida*. It has proven to be highly valuable, owing to beneficial natural qualities, which fit the requirements of biotech applications (Nikel & de Lorenzo, 2018). Yet, the antiviral arsenal of *P. putida* is scarcely described, and deletions of still unidentified phage defense systems may limit strain efficiency and survival, causing loss of resources in industry (Kamiński & Paczesny, 2024). The main factor hindering this research is the lack of phage libraries specific to this microbe. With the recent development of the CEPEST

collection, which contains a diverse list of *P. putida* phages (Brauer et al., 2024), we could study this area more extensively. Genomic engineering would benefit from a map of functional phage defense systems to make optimal changes and expand the practicality of this organism. Hence, this thesis set out to verify the functionality of three predicted anti-phage defense systems in *P. putida*.

1 LITERATURE REVIEW

1.1 INTRODUCTION TO ANTI-PHAGE DEFENSE SYSTEMS

1.1.1 Bacteria and phage interactions

Bacteria are found in diverse aquatic, seafloor sediment, and soil environments, where they are outnumbered by their viruses, bacteriophages (phages), with reported ratios of at least ten to one (Cobián Güemes et al., 2016). As bacteria constitute 15% of the global biomass, they are considerably involved in biogeochemical cycles (Bar-On et al., 2018), which can be modelled more accurately by accounting for the factors impacting bacterial processes. Primarily, the widespread existence of phages suggests strong interactions, which are estimated to cause 20-40% of bacterial lysis in surface waters (Suttle, 2007). In addition to their role as predators, phages that integrate into the genome can inhibit competitor microbes or introduce novel metabolic genes, allowing colonisation of novel niches. Phages can contribute a fitness advantage, and pose a selective pressure on bacteria through infections, exerting profound control over bacterial populations (Chevallereau et al., 2022). This dynamic interplay is a fundamental aspect of microbial research, describing the functioning of many ecosystems.

1.1.2 Bacterial anti-phage defense systems (classification)

Phages mainly achieve infection by two life cycles: the lytic, where entry and hijacking of host cell machinery allows replication and bacterial lysis to release progeny, and lysogenic, where incorporating into the host genome allows continuous survival as a prophage (Taylor et al., 2019). For bacteria to ensure their own survival under this evolutionary pressure, they encode multiple anti-phage defense systems to fight against phages. Notably, prophages often contribute to antiphage defense, as they carry various defensive loci, collectively termed superinfection exclusion (Sie) (Labrie et al., 2010). Recent scientific advancements have begun to unveil the complexity of bacterial defense, giving an insight into diverse functions and versatile compositions of different protein domains (Georjon & Bernheim, 2023). To describe anti-phage defense systems comprehensively, they are generally classified based on the mechanism of abrogating phage infection. The initial stages of infection, phage adsorption and genome entry into the bacterial cell, can already be halted by some defense systems. This is done either by blocking the entry receptor or stopping the phage genome injection. (Patel & Maxwell, 2023). If the breach nevertheless occurs, the next common mechanism is the degradation of viral nucleic acid. The diverse landscape of nucleases includes GIY-YIG, OLD, PD-(D/E)xK domain families, representing a group of enzymes with different features but the same defense strategy (Yang, 2011). Some of the most widespread defense systems in bacteria, restriction-modification (RM), and CRISPR-Cas, work in this way to prevent phage replication. While RM is an innate system that differentiates host from foreign DNA by methylation status, CRISPR-Cas is an adaptive system that destroys previously encountered genetic elements (Dimitriu et al., 2020). Another general antiphage strategy is the self-induced killing of the infected cell, thereby saving the nearby bacterial colony. These are termed abortive infection (Abi) systems, encoded by bacteria

or prophages, and consisting of at least two functional modules: a sensing and a killing one, although the killing mechanism can vary (Lopatina et al., 2020). Thus, phages have an impact on bacterial populations through predation and symbiosis as they ensure their survival.

1.1.3 Phage anti-defense systems and co-evolution

Furthermore, within this intricate interaction, phages encode anti-defense systems to overcome the barriers met throughout infection. These are proteins or RNAs that help evade bacterial detection mechanisms or attenuate their effects (Niault et al., 2025). The co-evolution can be illustrated by anti-RM and Acr (anti-CRISPR) being the most prevalent antidefense proteins in phages (Tesson et al., 2025), in correlation with the most common anti-phage defense systems. Studied in *Pseudomonas aeruginosa* prophages, the structures and mechanisms of Acr proteins vary greatly, but they generally bind to various parts of the CRISPR-Cas complex, inhibiting its activity. For example, some compete with target DNA for its interaction site on CRISPR, others through an allosteric mechanism, by occupying another site with an overlapping binding interface. While these prevent the recognition of phage DNA and the recruitment of a Cas helicase-nuclease, there are also Acr proteins that directly bind to Cas (Bondy-Denomy et al., 2015). The surface area required for exerting Arc function in different proteins ranges from a few amino acid residues (Maxwell et al., 2016) to oligomers (Wang et al., 2016). The variety of ways that phages overcome CRISPR-Cas seems to be interlinked with the diversity of these bacterial anti-phage defense systems, classified into 33 subtypes based on Cas protein differences and sequence divergence (Makarova et al., 2020). Such an extensive adaptive immunity would impact the evolution of anti-defense systems in phages, possibly explaining their ability to continue proliferating despite the prevalence of CRISPR-Cas systems (Bondy-Denomy et al., 2013).

Anti-RM protein function has mostly been studied in phages infecting *Escherichia coli*, specifically, ArdB interacts with the restrictase subunit of a type I RM complex to block its translocation along target phage DNA, therefore preventing endonuclease activity (Kudryavtseva et al., 2020). As ArdB inhibits the activity of different subtypes of type I RM systems, which target different sequences, the mechanism is not specific to the DNA recognition site of a complex. However, ArdB does not work against other types of RM systems, which are structurally different from type I RM. Moreover, enzymes targeting restrictase activity do not work against type II RM (Kudryavtseva et al., 2023). There must be a plethora of anti-defense proteins, attuned to escape the mechanism of each defense system to permit the ubiquitous existence of phages.

Similarly to the diversity of Acr proteins, studies of anti-RM proteins reveal how phages evolved to evade these widespread defenses, causing bacteria to further adapt to the pressure. For instance, RM type I-III systems cleave unmethylated foreign DNA, whereas resistant methylated phages that evade these defenses can then be targeted by RM type IV enzymes. They lack methyltransferase activity to target methylated phage DNA, cleaving it, and leave host DNA unmodified for self-protection (Dimitriu et al., 2020). The multiple lines of defense reiterating

the RM technique show its efficiency in bacterial defense and exemplify the ongoing co-evolution between bacteria and phages that diversifies both sides.

Other bacterial immune strategies can also be evaded by phages. The anti-phage defense system Pycsar in bacteria regulates cyclic nucleotide (cNMP) signalling, which in turn activates a downstream effector to trigger abortive infection. This causes membrane impairment or depletion of cellular NAD+, therefore stopping phage replication (Tal et al., 2021). In addition, Pycsar enzymes can cleave any cNMP, and for this function they are thought to have been co-opted by phages, which encode anti-Pycsar protein 1 (Apyc1). By inhibiting this signalling pathway, phages counteract Pycsar defense systems (Tesson et al., 2025). The origins of the Apyc1 protein showcase an example of the closely linked evolution of bacteria and phages.

1.1.4 Spread of anti-phage defense systems in bacterial genomes

The glimpses of the complicated makeup of bacterial defense set the incentive for studies to investigate still unvalidated systems, but effective exploration requires a strategic starting point. Genomic analysis revealed that defense systems co-localise in the genome in clusters known as defense islands (Makarova et al., 2011). This discovery has enabled experiments to link additional defense genes to already known ones based on the guilt-by-association principle (Doron et al., 2018). Finding them is a continuous process, as the model of pan-immunity suggests that a bacterial strain does not encode all defense systems, rather they are shared through horizontal gene transfer on a population level to overcome selection pressure, making the genomic defense composition of strains within a species subject to variability (Bernheim & Sorek, 2020). Observations show that variants of characterised systems, as well as those with still undefined mechanisms exist (Makarova et al., 2011). Further study and classification of genomic defense clusters could have an amplified impact on discovery, as this creates a base for studies to reveal novel candidates.

1.1.5 Phage defense locus prediction tools

Until recently, the discovery of defense systems has been restrained due to lack of accessibility to useful knowledge and prediction tools which could help select targets for investigation. However, the rapidly increasing capabilities of bioinformatics have improved this, producing a shift in experimental planning. Firstly, the PADLOC tool uses an ML model, which compares protein sequences to over 700 protein family components to identify putative defense systems (Payne et al., 2022). By ensuring systematic annotations for all system types, the tool expanded the scope of discovery, as previous software could only recognise genes belonging to a specific system type. This venture provides a reference point for information on novel defense systems, and existing ones, supporting functional studies (Payne et al., 2021). Another tool created to predict anti-phage defense systems is DefenseFinder, which similarly employs an ML model to compare protein sequences to homologs of known systems. The website also serves as a catalogue of existing studies that presents taxonomic distribution, links to AlphaFold structural predictions and consideration of potential complexes (Tesson et al., 2024). The outlined tools

improved accessibility to information and added novel systems to known classifications, however, there is a gap in expanding the knowledge beyond identified functions and protein homologs.

To investigate the broader landscape of bacterial defense, the focus was shifted away from defense islands in DefensePredictor. The tool employs an ML model to assess over 100 features of a gene's genomic context to predict whether they are consistent with a role in defense. As a result, novel systems can be identified in previously overlooked locations, including some with unique functions, with no detectable homology to previously known defense loci. In comparison, when analysing *E. coli* strains, PADLOC and DefenseFinder both identified less than 5% of the unique systems found by DefensePredictor. However, it performs worse when solely searching based on homology, and the performance on other species is still undetermined (DeWeirdt et al., 2025). This reflects the need for differently specialised tools to build upon each other for versatile discovery, which accelerates progress and broadens the picture of bacterial defense. Transferrable information should be consolidated across species to better understand less studied ones, later allowing to train other models to uncover unique defense systems. Therefore, analysis of a strain will benefit from both approaches and subsequent experimental validation.

1.2 THREE POTENTIAL ANTI-PHAGE DEFENSE SYSTEMS OF *PSEUDOMONAS PUTIDA* PaW85

1.2.1 HerA/DUF4297

The defense island principle and progress in prediction tools have enabled the compilation of a list of potential anti-phage defense systems, three of which were selected for the present work. Firstly, a study conducted an expanded computational search in *E. coli*, not limited to domain annotations usual for defense islands, aiming to identify unknown systems or those with functions typically not expected to be part of defense. The identified genes were validated using phage plaque assays on *E. coli* expressing the cloned candidate gene. It was found that HerA forms a complex with DUF4297, annotated as a helicase and nuclease respectively, together showing antiviral activity. The data showed that the complex is not enriched within prophages, therefore it is a dedicated bacterial system (Gao et al., 2020). According to prediction tools, *P. putida* PaW85 genes PP_4447 and PP_4448 respectively encode DUF4297 and HerA homolog proteins (Payne et al., 2022; Tesson et al., 2024). Further biochemical analysis in *E. coli* showed that HerA exhibits ATPase activity, and DUF4297 is a dsDNA nuclease with homology to the PD-(D/E)xK-family. Moreover, the structure of the complex in *E. coli* was elucidated as having 6 HerA molecules, and 12 molecules of DUF4297, with a combined molecular weight of ~1 MDa (An et al., 2024). The results show that the components only exert enzymatic activity within the complex, whereas individually both indicate extremely low activity. The complex assembly activates and stimulates the respective enzyme functions, which are degrading dsDNA, and possibly depleting ATP resources. This proposes the possibility of two prevalent immune

strategies being combined to provide concerted activities for a multi-layered defense (An et al., 2024).

The paper detailed the intermolecular contacts within the complex, particularly those mediated by the DUF4297 C-terminal domain (CTD), and demonstrated their significance by showing a loss of anti-phage function when mutations were introduced at these interfaces. It was previously known that complex formation induces dimerisation of the top layer of DUF4297 molecules, activating the N-terminal nuclease domain (NTD). However, the mechanisms driving this activation and the specific function of the CTD remained unknown (An et al., 2024). A recent study confirmed that DUF4297 may exist as dodecamers with monomeric nuclease domains. This indicated that enzyme activation is not driven by oligomerisation but by structural remodelling aided by intermolecular contacts. Association with HerA triggers a rearrangement of the DUF4297 CTD, which, through proximity, induces the formation of clamp-shaped DUF4297 NTD dimers, ready to cleave DNA (Rish et al., 2025). This role of the CTD aligns with observations that molecules in the bottom layer of DUF4297 lack mutual contacts, whereas molecules in the top layer – the site of dimerisation - interact through their CTDs. Furthermore, the CTD is connected to the NTD by a glycine residue, which provides the protein with conformational plasticity and flexible domain positioning (An et al., 2024). These structural insights highlight the functional importance of DUF4297 domain interactions. Together, the findings reveal how intermolecular interactions drive the structural rearrangement of the complex, enabling the active conformation essential for its function. Nevertheless, the trigger mechanisms for the nuclease activation during infection remain to be determined.

1.2.2 PD-T7-1

Another study employed functional metagenomics to detect systems not linked to defense islands in *E. coli* and found PD-T7-1 (Phage Defense against T7), a PD-(D/E)xK nuclease, to provide protection against phages. The PaW85 homolog is predicted to be encoded by the PP_0049 gene (Tesson et al., 2024). Experiments in *E. coli* showed that PD-T7-1 provides direct immunity, instead of abortive infection exhibited by other members of the superfamily, so its regulation and activation is distinct from other nucleases with the shared small motif (Vassallo et al., 2022). PD-(D/E)xK nucleases are in fact one of the most common domains in the unique proteins identified by DefensePredictor. Their predicted structures showed alignment of the catalytic motif, yet distinct additional domains could be found outside of it, creating diversity (DeWeirdt et al., 2025). Such domain conservation shows the effectiveness of this catalytic function, and structural variations corroborate likely different regulatory mechanisms of each nuclease.

1.2.3 Restriction-Modification Type II (RMII)

Lastly, the study includes a type II restriction-modification (RM) system, consisting of an endonuclease, which cleaves foreign DNA, and a methyltransferase protecting self-DNA with methylation (Dimitriu et al., 2020). RM systems are the most abundant antiviral systems, present in 83% of prokaryotic genomes (Tesson et al., 2022). In PaW85, the gene PP_3988 is predicted

to encode a restriction enzyme and PP_3989 the methylase (Tesson et al., 2024). In type II systems, the two enzymes encoded by the operon independently recognise the same 4-8 bp palindromic sequence (Pingoud, 2001). In this way, the target sequence is specific to the cell, allowing to differentiate between encountered DNA. The methyltransferase tags the sequence in self-DNA by methylating it, thereby preventing cleavage by the endonuclease, which targets and degrades unmethylated sequences originating from external sources (LeBlanc & Charles, 2022). Even though nucleases and helicases often have conserved domains, possibly facilitating the evolution of phage anti-defense systems, the prevalence of these enzymes in bacterial defense points to the efficiency of nucleic acid degradation in preventing infection (Mariano & Blower, 2023). Being the largest and most diverse group of the RM systems, RMII is likely to vary from organism to organism (Pingoud et al., 2014). Thus, as an important part of the immune strategy, RMII should be studied in each bacterium of interest.

1.2.4 *Pseudomonas putida* and its applications

Along with improved methods, a more comprehensive view of bacterial defense is emerging, and new findings about potentially involved genes are brought out. However, many systems studied in model organisms are only putatively annotated in others, and work is still required to understand the full potential of bacterial defense. *Pseudomonas putida* is a Gram-negative soil bacterium, whose ability to degrade toxic compounds has led to biotechnology applications, continuously growing with improved understanding of the genome (Belda et al., 2016). Among these is plant rhizosphere colonisation to improve growth and protect against pathogens (Planchamp et al., 2015). Natural ability to degrade toxic compounds could be leveraged in biorefineries for lignin byproduct degradation (Nikel & de Lorenzo, 2018). Additionally, the innate metabolism offers a favourable platform for inserting heterologous pathways for synthesis of valuable compounds (de Lorenzo et al., 2024). Together, the larger implications of sustainable production, fighting climate change with renewable energy and improving agriculture, provide a compelling argument to study *P. putida* further for fruitful implementation.

1.2.5 Use of genomic information in biotechnology

In biotechnological applications, bacterial strains are often improved by deleting competing or non-essential genes from their genomes, resulting in faster growth rates or higher production yields (LeBlanc & Charles, 2022). For instance, deleting 4.3% of the *P. putida* KT2440 genome produced a strain with increased availability of ATP and NAD(P)H, faster bacterial growth, reduced sensitivity to endogenous oxidative stress, and improved expression of heterologous genes (Martínez-García et al., 2014). Creating reduced strains requires methods that determine gene essentiality, such as *in silico* modelling, which complements experimental approaches (LeBlanc & Charles, 2022). Genome-scale metabolic models are widely used in industrial and environmental applications to evaluate the impact of changes in an organism's metabolic network. A study on redirecting metabolic fluxes for bioplastic precursor production in *P. putida* strain KT2440 highlighted that incomplete gene annotations can hinder both the construction and accuracy of these models. Unidentified genes with critical roles may exist, while pinpointing

redundant genes could reveal targets for pathway optimisation (Puchałka et al., 2008). These challenges extend to assessing metabolic behaviour under various stress conditions, including phage infections. Identifying the lethality of specific gene deletions is particularly relevant for anti-phage defense systems, as phage infections during industrial fermentations often result in reduced productivity or complete batch failure (Kamiński & Paczesny, 2024). Even determining that a gene is not involved in defense or its function is redundant could help to reduce unnecessary parts of engineered biochemical pathways. A disadvantage of current computational approaches is the lack of gene annotations in non-model organisms, which complicates predictions, potentially leading to less efficient applications, and perpetuating reliance on costly, time-consuming experiments (LeBlanc & Charles, 2022). Therefore, functional studies of potential defense genes in *P. putida* are essential to refine metabolic models and advance biotechnological endeavours.

1.2.6 Relevance of anti-phage defense systems in applications

Despite growing interest in biotechnology, the anti-phage defense systems of *P. putida* remain understudied. In contrast, extensive research on other organisms, such as *E. coli*, has demonstrated promising outcomes, underscoring the industrial importance of anti-phage defense systems. For example, the discovery of the Ssp PT (ssDNA phosphorothioate) modification system with functions analogous to RM systems revealed the existence of unusual defense. The system composes of an endonuclease which introduces nicks to phage DNA, rather than typical cleavage, showing a different mechanism from other DNA-targeting defense systems. Other proteins in the system replace an oxygen atom by sulfur in the backbone of a DNA strand, creating sequence-specific single-stranded modifications to protect self-DNA, therefore helping to target non-modified phage DNA (Xiong et al., 2020). In contrast to other similar modification-based systems, where disruption in this function causes host DNA targeting by the nuclease of the system, Ssp PT exerts no toxicity on host cells lacking the modifying proteins. This prompted researchers to engineer industrially relevant *E. coli* strains to contain this defense system, increasing the resistance to various phages, while reducing risk to cell viability. The solution could mitigate issues like failed fermentation and persistent facility contamination, preventing significant financial loss (Zou et al., 2022). Studying a novel defense system uncovered unknown mechanisms, which could then be leveraged for more optimal genomic changes, improving the efficiency of industrial processes.

To conduct similar research on *P. putida*, the choice of bacterial strain must be considered. According to the pan-immunity model, the content of defense systems within a strain is dynamic, with cells acquiring and losing genes over time from the surrounding population. Consequently, closely related strains of a species may have drastically different compositions of defense systems (Bernheim & Sorek, 2020). While studying a single strain does not represent the entire species, this work focuses on *P. putida* KT2440 due to its importance in biotechnology. Given the unique niche of *P. putida* in potential applications, a deeper understanding of its particular set

of anti-phage defense systems is crucial to generate genomic insights needed for successful manipulation and optimisation.

1.2.7 The CEPEST collection of *P. putida* phages

A considerable limitation for the experimental verification of anti-phage genes is the extensiveness of the available phage library. It is likely for false negatives to occur in assays if a defense system is specific to a certain type of phage absent from the test set (Doron et al., 2018). The phages isolated for *P. putida* have been limited, leading to gaps in the understanding of its defense (Magill et al., 2017). Moreover, few phages particular to the biotechnologically relevant KT2440 strain have been reported until recently (Jaryenneh et al., 2023; Ngiam et al., 2022). This thesis benefits from CEPEST, the largest environmental phage collection for *P. putida* to date. The phages were isolated from muddy water and soil samples using the PaW85 strain, which is isogenic to KT2440, but in this case weakened by the deletion of 13 toxin-antitoxin systems and 4 prophages. The resulting collection comprised 67 tailed, double-stranded DNA phages belonging to the order *Caudovirales*. These phages demonstrated high host specificity, as most were unable to infect other *P. putida* strains or *Pseudomonas* species. Sequence analysis using VIRIDIC grouped the phages into nine genus clusters based on sequence similarity. This analysis revealed novel clusters, increasing the diversity of known phage genomes (Brauer et al., 2024). As the collection expanded over the course of this work, the rich assortment of phages provided new opportunities to test the defensive role of implicated genes that had not been possible before.

2 THE AIM OF THE THESIS

- The first aim of this thesis is to verify the functionalities of three predicted anti-phage defense systems in *P. putida*.
- Thereafter, the 2nd aim was to validate the effects of the deletion strain of HerA/DUF4297 by complementation
- The third aim was to investigate via active site mutations whether the *P. putida* DUF4297 homolog could function analogously to the respective *E. coli* protein.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Bacterial strains, plasmids and media

LB (Lysogeny broth) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) was used for bacterial culture. LB agar (1.5% agar) and LB soft agar (0.3% agar) were used. LB soft agar was supplemented with 10 mM $CaCl_2$. Ciprofloxacin (Cip) at a final concentration of 0.03 μg/ml was added to LB agar for phage susceptibility assays.

Strains used in cloning and phage susceptibility assays are listed in Table 1.

Table 1.

Strain	Description	Source
Escherichia coli DH5α λpir	*endA1 hsdR17 glnV44 (= supE44) thi-1 recA1 gyrA96 relA1 φ80dlacΔ(lacZ)M15 Δ(lacZYA-argF)U169 zdg-232::Tn10 uidA::pir+*	(Platt et al., 2000)
Pseudomonas putida PaW85	Wild-type, isogenic to KT2440	(Bayley et al., 1977)
PaW85 ΔPP_0049	*P. putida* with a deletion of PD-T7-1	This project
PaW85 ΔPP_4447-4448	*P. putida* with a deletion of HerA/DUF4297	This project
PaW85 ΔPP_3988-3989	*P. putida* with a deletion of RMII	This project
PaW85 ΔPP_4447-4448 + HerA/DUF4297	*P. putida* with a deletion of HerA/DUF4297 complemented with HerA/DUF4297 in mini-Tn7	This project
PaW85 ΔPP_4447-4448 + HerA/DUF4297(Q54A K56A)	*P. putida* with a deletion of HerA/DUF4297 complemented with HerA/DUF4297$_{Q54A\ K56A}$ in mini-Tn7	This project

Plasmids used for bacterial cloning are listed in Table 2.

Table 2.

Plasmid	Description	Source
pSNW2	Used for genomic integration of constructed loci	(Volke et al., 2020)
pSNW2del-PD-T7-1	Contains PD-T7-1 deletion locus	This project
pSNW2del-HerA/DUF4297	Contains HerA/DUF4297 deletion locus	This project
pSNW2del-RMII	Contains RMII deletion locus	This project
pSW-I-SceI	Contains the I-SceI nuclease to remove co-integrate	(Martínez-García & de Lorenzo, 2011)
pUXBF13	Helper plasmid which provides Tn7 transposition function	(Bao et al., 1991)
pGP704L-miniTn7Gm	Mini-Tn7 plasmid used to insert complementation locus into Tn7 genomic integration site	(Jakovleva et al., 2012)
pGP704L-miniTn7Gm-HerA/DUF4297	Used for complementation with HerA/DUF4297 system	This project
pGP704L-miniTn7Gm-HerA/DUF4297(Q54A K56A)	Used for complementation with an active site mutation in the HerA/DUF4297(Q54A K56A) system	This project

3.1.2 Phages

The phages used in phage susceptibility assays to infect bacterial strains are listed in Table 3.

Table 3.

Phage	Species	Source
1A	Amme-3	(Brauer et al., 2024)
1B	KoPa-4	
1C	NoPa-1	
1D	Kassivere	
1E	Roomu-2	
1F	NoPa-2	
2A	IPa-1	

2B	Vasula	
3A	Illi-2	
3B	Amme-1	
3C	Lauda	
3D	Peetri	
4A	Emajogi	
4B	Luke-2	
5A	ErraM	
5B	Laguja-2	
5B	Illi-1	
5B	KK3	
5B	Konnatiik	
5B	Laguja-5	
5B	Luutsna-1	
5B	Luutsna-3	
5B	Luutsna-6	
5B	Mora-3	
5B	Mora-5	
5B	Paidla	
5B	Pori-2	
5B	SaviPeeda	
5B	Villemi	
5B	Ihaste	
5B	Kaagvere	
5B	Urmase-lomp	
5B	Viia-tiik	
5C	BotAed	
6A	Luke-3	
7A	Kallioja	
7B	Kompost-2	
7C	ToKo-1	
8A	Kurepalu-1	
9A	Kurepalu-2	
9B	Mudajogi	
10A	TPkomp II-1	Unpublished
11A	Spalona	
12A	Zukowo	
13A	KeKo-2	
?	Pan16	

17D	Pan31cl	
17D	Pan25op	
17D	Pan23	
17D	Ülenurme4H	
17D	Ülenurme4S	
17D	Ülenurme5S	
17D	Ülenurme5H	

3.1.3 DNA cloning

Primers used in mutant construction strains are listed in Table 4.

Table 4.

Primer name	Sequence	Restriction Enzymes	Function
30 del0049FrontFwSal	CATgtcgaCGATTCACTCGTCTGATGCTC	SalI	PD-T7-1 deletion
31 del0049FrontRevLong	TCTACCTGCGTGGCAACGATCGGTGGAGGTACCTATTGAAGAAG		
32 del0049RearFw	ATCGTTGCCACGCAGGTAGA		
33 del0049RearRevEco	TAgaattCCCATAATAATTCCGGTACAGTACATGAC	EcoRI	
36 del3988-89FrontFwXba	gatctagaTGGACGGCGTAATCCTGTTC	XbaI	RMII deletion
37 del3988-89FrontRevLong	CGTCTTTGACGTCCATATCGTGATGGTTTTTGCCTGTAAAAAAATGC		
38 del3988-89RearFw	ACGATATGGACGTCAAAGACG		
39 del3988-89RearRevEco	TAGAATTCGCACACAGGTCAATGTTTACTAC	EcoRI	

42 del4447-48FrontFwEco	ATgaattcATACCAACACCTCTGCGATG	EcoRI	HerA/DUF4297 deletion
43 del4447-48FrontRevLong	TCTCAGATGAGCAGCAGTGCTAAGACATCCTATTAAAAATGGCGTCG		
44 del4447-48RearFw	GCACTGCTGCTCATCTGAGA		
45 del4447-48RearRevSal	TATGTCGACGGCAAGAATTGGTGAGTGAGC	SalI	
77 Tn7R109sisse	CTGAAATCAGTCCAGTTATGCTG		Verifying the correct orientation of fragment inserted into pGP704LminiTn7Gm
78 Tn7Gmlopp	CCTAACAATTCGTTCAAGCCGA		
84 HerADUF Tn7 Fw Sma	ACCCGGGATACCAACACCTCTGCGATG	SmaI	Constructing DUF4297 active site mutation
85 HerADUF Tn7 Rev NotI	ATGCGGCCGCCATTACGGAAGCCCTACCTTC	NotII	
88 seq HerDUF 3	CGAGAAAGCATATCAAACGGA		Checking HerA/DUF4297 transformation
100 DUF4297 I53 Rev2	gatgtatcgctcgctggaaac		Constructing DUF4297 active site mutation
101 DUF4297 Q54AK56A Fw2	tttccagcgagcgatacatcGCGTGCGCTtactatgcagctacagagtac		
Tn7R109	CAGCATAACTGG ACTGATTTCAG		Checking mini-Tn7 integration
Tn7GlmS	AATCTGGCCAAG TCGGTGAC		
Fw	GTAAAACGACGGCCAGT		Checking deletion fragment insertions in pSNW2
Rev	CAGGAAACAGCTATGAC		

All clonings were performed with Thermo Fisher FastDigest restriction enzymes and T4 DNA ligase according to the manufacturer's protocols.

To construct pSNW2-based deletion locus plasmids, regions flanking the deletion site were amplified using oligo pairs 30+31 and 32+33 (for ΔPP_0049), 36+37 and 38+39 (for ΔPP_3988-3989) or 42+43 and 44+45 (for ΔPP_4447-4448). The two PCR fragments for each deletion were joined by overlap extension PCR using the two outer oligonucleotides as primers. The obtained fragments were cloned into pSNW2 using SalI and EcoRI (ΔPP_0049 and ΔPP_4447-4448) or XbaI and EcoRI (ΔPP_3988-3989).

To construct the HerA/DUF4297 complementation plasmid, the genomic locus was amplified using oligo pair 84+85. The fragment was cloned into pGP704LminiTn7Gm using SmaI and NotI.

The complementation with an active site mutant was done by amplifying the two halves of the HerA/DUF4297 genomic locus with oligo pairs 84+100 and 85+101. The fragments were joined by overlap extension PCR using the oligos 84+85. DNA was purified from the agarose gel using the MicroElute Cycle-Pure & Gel Extraction Kit (Omega Bio-tek) following the manufacturer's instructions. The fragment was cloned into pGP704LminiTn7Gm using SmaI and NotI.

The plasmids were transformed into *E. coli* by electroporation and purified using the FavorPrep Plasmid DNA Extraction Mini kit, (Favorgen) per manufacturer's instructions.

All obtained plasmids were verified by sequencing at the University of Tartu Core Facility of Genomics.

3.1.4 Engineering *Pseudomonas putida* mutant strains

The method of Martínez-García & de Lorenzo (2011) was used to delete genomic loci from *P. putida.* Briefly, the deletion locus was introduced to *P. putida* on the pSNW2 plasmid, which enters the genome at the deletion site by homologous recombination, forming a cointegrate. Next, the pSW-I-SceI plasmid was introduced, from which the I-SceI nuclease expression was induced to generate double-stranded breaks at the integrated plasmid and thus excise from the genome. Thereafter, PCR with the outer deletion oligos was performed to verify whether the original locus or the deletion locus remained in the genome. Clones that had lost the wild-type locus were chosen and stored in glycerol stocks at -80 °C.

To construct the Tn7 complementation strains, the *P. putida* ΔHerA/DUF strain was co-transformed with two suicide plasmids. A pGP704L derivate contained mini-Tn7 carrying the studied loci and a Gentamycin resistance gene, and pUXBF13 contained the transposase gene for mini-Tn7. Clones with successful mini-Tn7 integration were selected on LB agar media containing 10 μg/ml Gentamycin.

3.1.5 Phage susceptibility assays

Bacterial culture was grown overnight and refreshed into new LB medium to allow exponential growth for 4 hours. To prepare double-layer agar plates, 200 µl of bacterial culture and 50 µl $CaCl_2$ were mixed with 5 ml soft LB agar and poured onto solid LB Cip medium. The assays on stationary phase cells were otherwise identical, except that 75 µl of overnight culture was used in the mix. The starting phage titre was 10^8 CFU/ml, from which 5 serial 10-fold dilutions were made in SM buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 8 mM NaCl, 8mM $MgSO_4$, 0.01% gelatin). 1.5 µl drops of each dilution were spotted on the double layer agar plates and incubated overnight in 20°C. Phage susceptibility assays were repeated three times.

3.2 RESULTS

3.2.1 Phage susceptibility of strains lacking predicted defense loci

Bacterial anti-phage defense system prediction tools consulted to choose candidates showed overlaps in predicting the final systems to have a role in defense. To test whether the three potential defense systems protect *P. putida* against phages, I first engineered individual deletion strains missing each of the hypothetical defense systems. Using the method of (Martínez-García & de Lorenzo, 2011), I created the deletion versions of the genomic loci by overlap extension PCR, then cloned them into pSNW for the homologous recombination-based deletion protocol. I verified that the wild-type locus had been replaced by the deletion locus with PCR and finally generated 2 clones of each system's deletion strain. This enabled me to test the effect of deletion on susceptibility to phages, by comparing phage infection efficiencies on each deletion strain with the wild-type.

Any increased sensitivity to phages of the deletion mutants was determined by an increase in plaques within higher phage dilutions. Throughout my research, I tested a total of 53 phages from the CEPEST collection, categorised into 14 genus clusters. The PD-T7-1 and RMII deletion mutants did not demonstrate consistently altered susceptibility to the tested phages, as most of them produced no effect, and the sparsely occurring changes were only observed in one out of three repeats per phage. Although both mutants showed susceptibility to some phages in two experiments, these results could not be reproduced in the third repeat. In contrast, the HerA/DUF4297 deletion mutant exhibited significantly higher susceptibility to a variety of phages, with these results consistently repeated across three experiments. An example of a phage susceptibility assay can be seen in Figure 1.

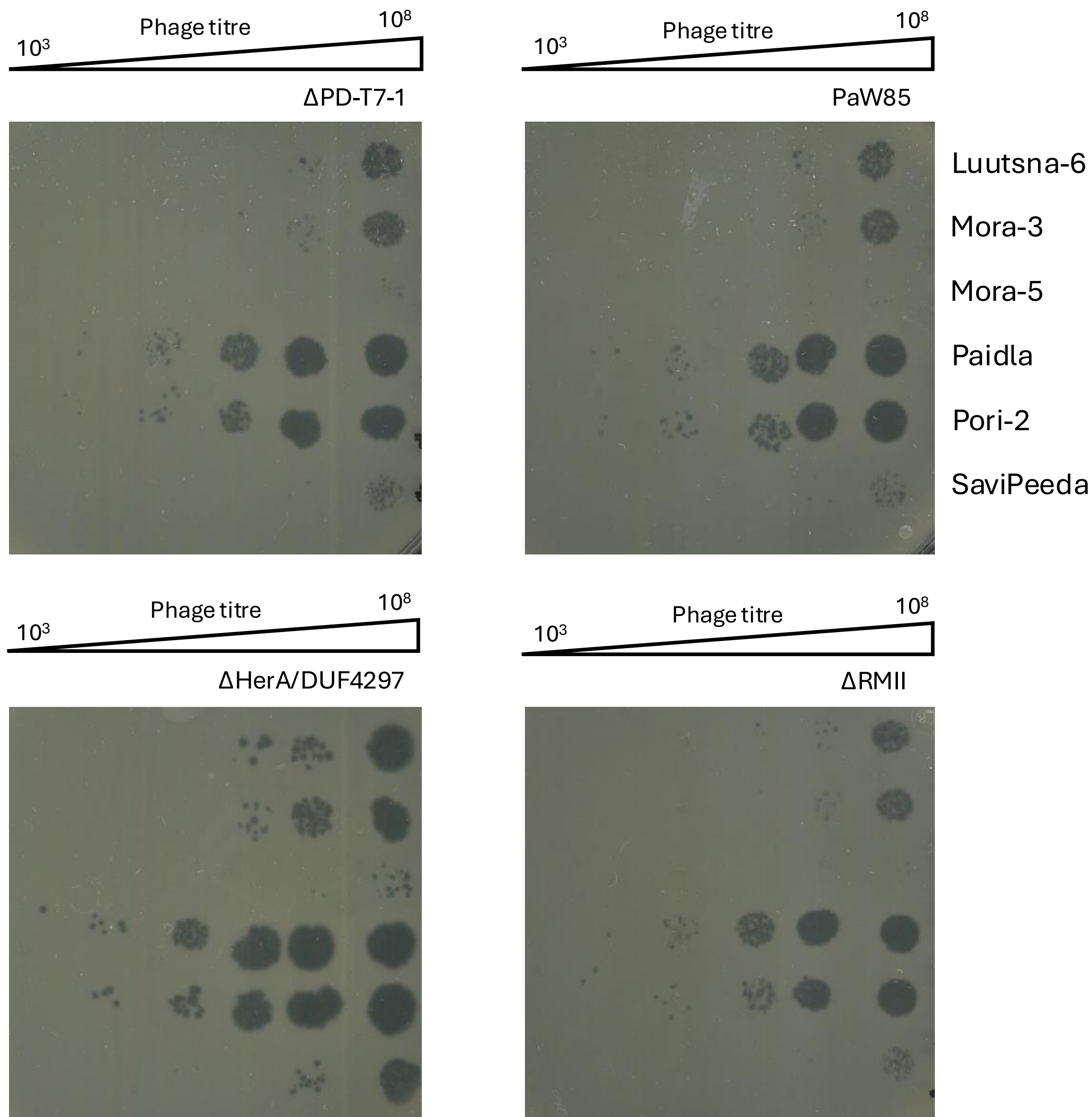

Figure 1. Phage plaque assay using CEPEST phages from species cluster 5B, listed on the right.

The increased infection efficiency on the ΔHerA/DUF4297 strain in comparison to PaW85 could most frequently be seen by phages belonging to genus cluster 1, 5 and 17. In particular, the species cluster 5B figured most prominently. Figure 2 summarises the observed infection efficiency of the mutants across three repeats with each phage. Because of the consistent observations of increased mutant susceptibility, HerA/DUF4297 was a subject of further experiments as a plausible candidate for anti-phage defense function.

Effect
100x
10x
0x
-10x

		Predicted defense system		
Species	Name	PD-T7-1	HerA/DUF4297	RMII
1A	Amme-3			
1B	KoPa-4			
1C	NoPa-1			
1D	Kassivere			
1E	Roomu-2			
1F	NoPa-2			
2A	IPa-1			
2B	Vasula			
3A	Illi-2			
3B	Amme-1			
3C	Lauda			
3D	Peetri			
4A	Emajogi			
4B	Luke-2			
5A	ErraM			
5B	Laguja-2			
5B	Illi-1			
5B	KK3			
5B	Konnatiik			
5B	Laguja-5			
5B	Luutsna-1			
5B	Luutsna-3			
5B	Luutsna-6			
5B	Mora-3			
5B	Mora-5			
5B	Paidla			

		Predicted defense system		
Species	Name	PD-T7-1	HerA/DUF4297	RMII
5B	Pori-2			
5B	SaviPeeda			
5B	Villemi			
5B	Ihaste			
5B	Kaagvere			
5B	Urmase-lomp			
5B	Viia-tiik			
5C	BotAed			
6A	Luke-3			
7A	Kallioja			
7B	Kompost-2			
7C	ToKo-1			
8A	Kurepalu-1			
9A	Kurepalu-2			
9B	Mudajogi			
10A	TPkomp II-1			
11A	Spalona			
12A	Zukowo			
13A	KeKo-2			
?	Pan16			
17D	Pan31cl			
17D	Pan25op			
17D	Pan23			
17D	Ülenurme4H			
17D	Ülenurme4S			
17D	Ülenurme5S			
17D	Ülenurme5H			

Figure 2. Phage susceptibility heatmap for ΔPD-T7-1, ΔHerA/DUF, ΔRMII with CEPEST phages, with VIRIDIC species clusters specified. The columns are three defense system deletion mutants. The cells for each defense system represent three independent repeats of assays conducted with each phage on the deletion mutant of the system. The effect corresponds to a change in susceptibility from the wild-type, where positive values indicate more efficient infection of the mutant. Cells are coloured when a change is observed in at least two repeats. The white cells mean no effect was observed, or it was only present in one repeat.

3.2.2 Phage susceptibility of ΔHerA/DUF4297

The consistent ΔHerA/DUF4297 phage susceptibility was followed up by verifying that the cause of observed effects is loss of function. To ensure that the increased infection was not due to potential unintended changes during the deletion locus construction, I created a complementation strain. The HerA/DUF4297 system along with ~500bp of its promoter region was re-inserted into a specific integration site in the genome on mini-Tn7. Infection efficiency was tested with phages that produced more plaques on the ΔHerA/DUF4297 strain that the wild-type, from genus clusters 1 and 5. Indeed, the transposon HerA/DUF4297 complementation strain behaved like the wild-type rather than the deletion strain (Figure 3A-C), indicating that the loss of HerA/DUF4297 was the reason behind the increased phage susceptibility. The deletion mutant was not obstructed by any random mutations that would impact its behaviour, and HerA/DUF4297 function is part of defense.

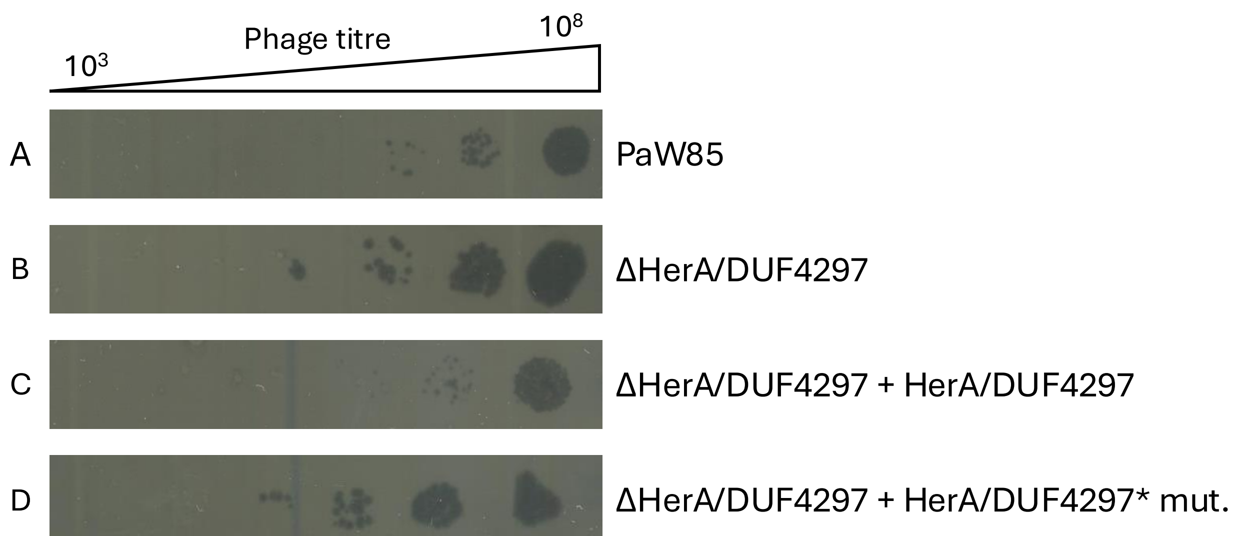

Figure 3. Phage plaque assay with the G5B phage Laguja-2 infecting PaW85, HerA/DUF4297 deletion mutant, complementation and complementation with an active site mutation.

3.2.3 Verifying HerA/DUF4297 function

In addition, we wanted to probe if the mechanism of the *P. putida* HerA/DUF4297 system might be similar to the *E. coli* homolog's. Mutating amino acids Q53 and K55 in the DUF4297 nuclease was shown to abrogate HerA/DUF4297-mediated defense (An et al., 2024). Sequence and structural model comparison identified the corresponding amino acids in *P. putida* to be in positions 54 and 56, respectively. By mutating them, it can be seen if the defensive function of the protein is inactivated. As the UniProt Align tool results show a sequence identity of 31.73% for DUF4297 in the two bacteria, additional considerations, like active site residue and structural model comparison, are needed to confirm conservation. The alignment of the DUF4297 protein's active site residues in *E. coli* with its homolog in *P. putida* is an indication that this key region is conserved. The residues identified in *P. putida* as corresponding to the ones in *E. coli* through

sequence alignment are consistent with the DUF4297 crystal structure in *E. coli* and structural model in *P. putida*, as they are positioned similarly in the N-termini (Figure 4 A, B). I created active site mutations of DUF4297 by substituting both glutamine in position 54 and lysine in 56 with alanine, based on the An et al. (2024) study on *E. coli*. Containing this mutation, the HerA/DUF4297 system along with ~500bp of its promoter region was cloned into mini-Tn7 and re-inserted into the ΔHerA/DUF4297 strain. Infection efficiency was tested with phages from genus clusters 1 and 5 that made the ΔHerA/DUF4297 strain more susceptible than the wild-type. As expected, the locus containing the Q54AK56A mutant version of DUF4297 failed to protect *P. putida* against phage infection and the phenotype was similar to the complete deletion of HerA/DUF4297 (Figure 3 D vs A-C), indicating that the HerA/DUF4297 system in *P. putida* most likely carries out DNA degradation, like the *E. coli* homolog.

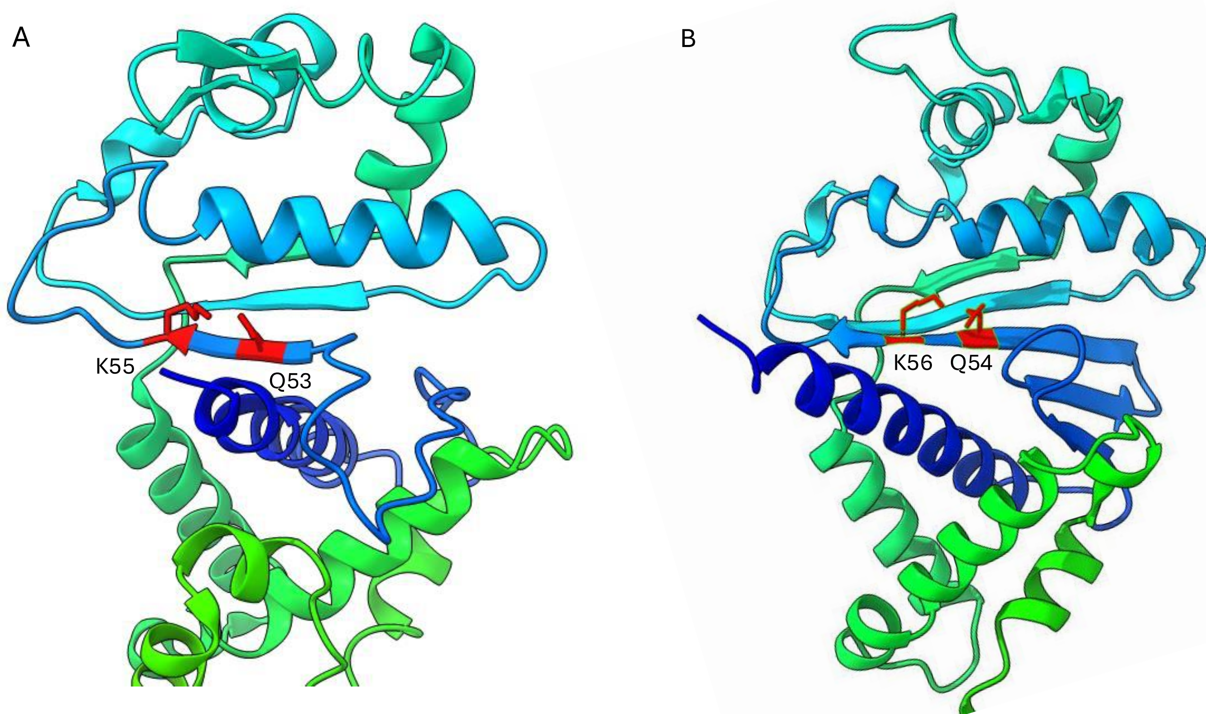

Figure 4. A) Crystal structure of DUF4297 in *E. coli* (PDB code: 8ZGI) (Tang et al., 2025) and (B) AlphaFold structural model of DUF4297 in *P. putida,* with active site residues highlighted in red.

3.2.4 Increased protection of HerA/DUF4297 complementation strain

In some of these experiments using strains associated with the HerA/DUF4297 system, the complementation strain showed an even stronger protection against phages than PaW85, as less plaques could be seen (Figure 3 A vs C). It is possible that the expression levels of HerA/DUF4297 are higher in the complementation strain even though the genes are expressed from their native promoter region. One hypothesis is that since mini-Tn7 always enters the chromosome close to the origin of replication (Peters, 2014) and the infection assays were performed with exponentially growing bacteria, the effective copy number of the *herA/DUF*

genes is increased (Teufel et al., 2023). This prompted to repeat the experiment with stationary phase bacteria, where we expect a uniform copy number of 1 for the whole genome. Indeed, on the stationary phase cells, both clones of the complementation strain had the same susceptibility to phages as the wild-type (Figure 5).

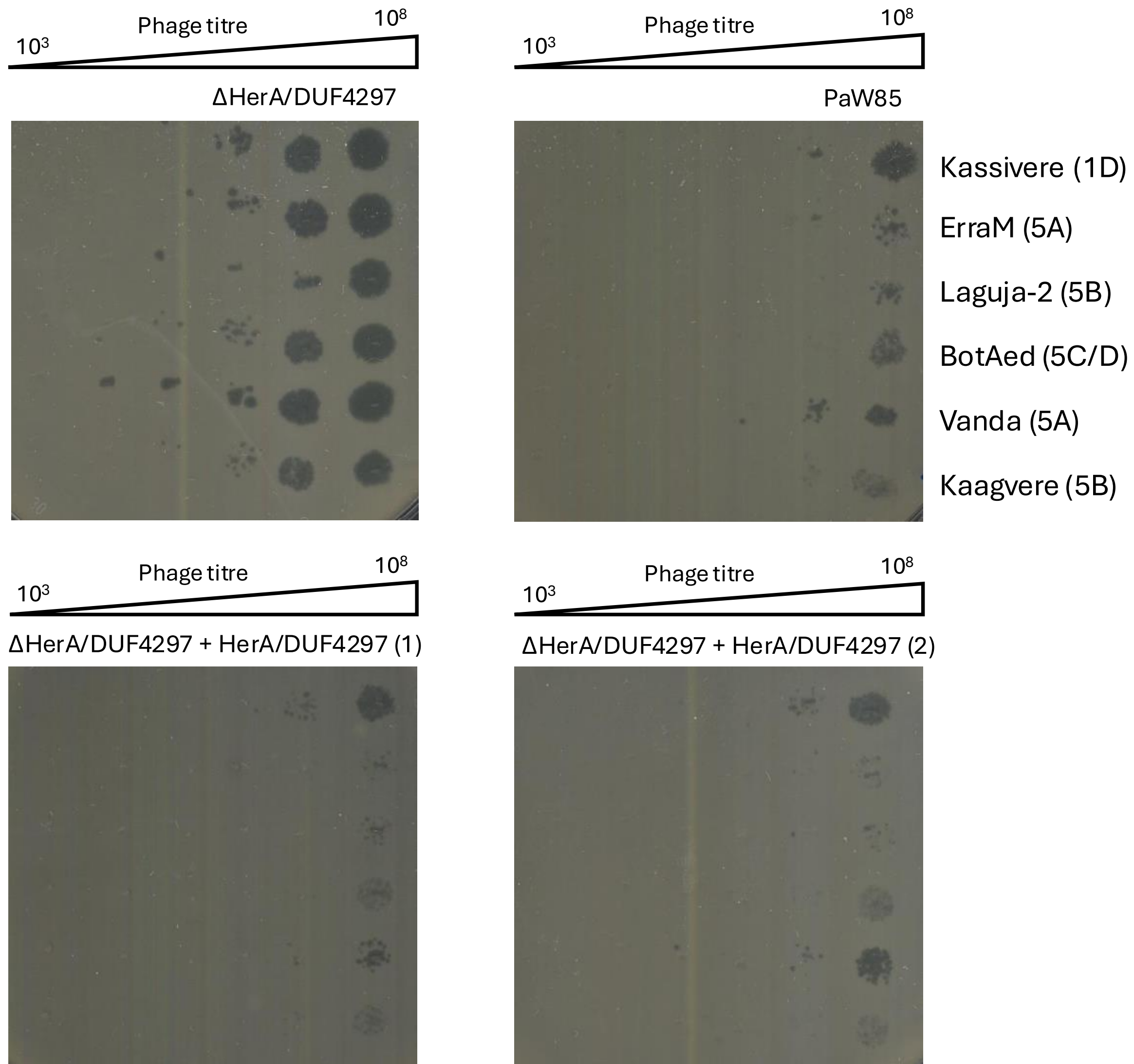

Figure 5. Phage plaque assay with stationary phase bacteria using phages from the CEPEST collection, listed on the right.

3.3 DISCUSSION

3.3.1 Unverified defensive role of the PD-T7-1 and RMII systems

This project approached the verification of predicted anti-phage defense systems PD-T7-1, RMII and HerA/DUF4297, by constructing individual deletion strains for each of them. The success in deleting these loci shows that they are not essential for *P. putida* and laid the groundwork for phage susceptibility assays. The deletion mutants for PD-T7-1 and RMII systems did not show clearly higher susceptibility than the wild-type, therefore, their role in anti-phage defense in *P. putida* could not be verified. However, it would be premature to classify them as inactive, as infection with some phages did show an increase in mutant cell death in one or two repeats. Because of the sparsity of phages causing effect and the lack of reproducibility, these systems were not pursued in further hypothesis testing. Additionally, the host used to isolate phages for the CEPEST collection was *P. putida* PaW85 Δ13TAΔ4φ, which lacks 13 toxin-antitoxin loci and 4 cryptic prophages, but does contain HerA/DUF4297, PD-T7-1 and RMII (Brauer et al., 2024). This means that the panel of tested phages is inherently able to overcome all three investigated defense systems and thus, our experimental setup was only able to detect differences in cases where the system provides incomplete protection. It is possible that environmental phages exist that are strongly inhibited by any of the studied systems and could not therefore be isolated.

Although the phages tested do not represent a comprehensive list of all existing *P. putida* phages in the environment, our collection nevertheless contains a diverse range of species. Therefore, the lack of substantially increased phage susceptibility of ΔPD-T7-1 and ΔRMII could indicate a narrow phage specificity if these systems provide anti-phage defense in *P. putida*. With RM systems being abundantly encoded in bacterial genomes (Tesson et al., 2022), they could each have evolved to target specific phages, rather than a wide range, together providing a solid defense strategy. Especially with RMII systems being the most diverse of the RM types (Pingoud et al., 2014), the individual specificity could vary based on the constantly evolving strategy attuned to interactions with phages. This would make it difficult to verify defensive function if phages specific to a given system are rare at a given point in the co-evolution. Similarly, PD-T7-1 is part of the common PD-(D/E)xK nucleases which are very diverse (DeWeirdt et al., 2025), making it likely that the activation of each nuclease is specific. Otherwise, there could be redundancy between different enzymes that protect against the same phages, causing a limitation in validating defensive function in a single-system deletion mutant. Then, other systems could be compensating for the absence of a defense system. The verification of potential defense systems is highly dependent on the diversity of the phage collection, which poses an experimental limitation. If PD-T7-1 and RMII have a role in defense in *P. putida*, at present the phages specific to these systems might not be in the collection. The expansion of the CEPEST collection using a host strain lacking potential defense systems could present novel opportunities for validating the defensive function of these predicted systems. Therefore, at this point, PD-T7-1 and RMII were not studied further and the thesis focused on the antiphage effects of the HerA/DUF4297 system.

3.3.2 Defensive role of HerA/DUF4297

The consistent increased susceptibility of the HerA/DUF4297 deletion mutant to numerous phages and follow-up experiments showed that this system has a role in anti-phage defense. The complementation strain verified that the deletion strain did not contain random mutations which could impact the phenotype in phage infection, and the observed effects were representative of the loss of HerA/DUF4297. This allowed to investigate the function exerted by the system, by complementation with an active site mutation. This strain had the same susceptibility to phages as the full deletion strain, indicating that the enzymatic function is crucial in the defensive effects. As the residues mutated were based on the study characterising DUF4297 in *E. coli* as a nuclease (An et al., 2024), this function is most likely the same in *P. putida*.

From the phages causing stronger infection in the deletion mutant, those from genus cluster 5 are overrepresented. They are also the most abundant in the CEPEST collection, and phage isolations without an enrichment step indicate that they are indeed prevalent in environmental samples (Brauer et al., 2024), making the protection provided by the HerA/DUF4297 system stand out in importance. Since clusters 1 and 5 are the most prevalent in susceptibility assays for the HerA/DUF4297 deletion mutant, it could be a system with specificity for the main phages threatening PaW85 survival.

Successful biotechnology applications depend on strain-specific genomic information to make accurate changes for productivity, while maintaining cell viability. The ancestor strain of PaW85, mt-2, shows higher resistance to phages of genus cluster 5. This is caused by the TOL plasmid pWW0, absent from PaW85 (Brauer et al., 2024). While colonising the environment, mt-2 likely adapted a strong defensive strategy against the most prevalent phages. As PaW85 is particularly relevant for research and industrial purposes, determining its specific immune components informs genomic engineering decisions. Specifically, the protection of the HerA/DUF4297 system is important, as PaW85 lacking the TOL plasmid could become more susceptible if another line of defense is lost.

The improved phenotype of the HerA/DUF4297 complementation strain to the wild-type was unexpected. A hypothesis related to different gene expression due to the different genomic positions of the loci was tested. Due to DNA replication time being longer than cell duplication time, bacteria overlap several replication rounds, by starting a new one before the previous one is finished. Therefore, genes located closer to the origin of replication (oriC) are copied the earliest, resulting in increased copy number and expression for a longer time (Teufel et al., 2023). As the distinct effects were seen with exponential phase cells, the phage susceptibility was tested again with stationary phase strains. Indeed, the complementation strain showed the same susceptibility to phages as the wild-type. The Tn7 integration site, used to reinstate the deleted HerA/DUF4297, is at the 3' end of the *glmS* gene (Peters, 2014), which is found approximately 11.5 kbp away from the oriC, while the *duf4297*/*herA* locus is located ~1.1 Mbp from oriC, likely causing higher expression of the system in the complementation strain in exponential phase. This elicited stronger protection against phages than PaW85 harbouring the locus in the

usual genomic location. These results also indicate that the HerA/DUF4297-mediated defense is not saturated in its native form and synthetic overexpression could possibly be utilized as a way of artificially increasing phage tolerance of PaW85.

3.3.3 Future directions

Experimental verification of anti-phage defense systems sets the basis for further characterisation. As researchers are still uncovering parts of bacterial immunity beyond the most frequently studied systems, information on mechanisms is often lacking. The recent discovery of the structural rearrangement needed for the activation of the HerA/DUF4297 system in *E. coli* (Rish et al., 2025) could be a basis to determine the activation of the complex in *P. putida*. Based on my work, the function of this system in both bacteria is most likely the same, but the variation in residues outside of the active site could mean different regulatory mechanisms. Conserved genes with anti-phage defense functions may work differently from organism to organism, as selective pressure from phages specific to a colonisation niche would cause each bacterium to adapt uniquely (Tesson et al., 2022). Therefore, the sensing of phage infection and triggering of the HerA/DUF4297 response should be studied in *P. putida*.

The activation and regulation of the response to phage infection could provide a more holistic view of the cell's behaviour. For example, synergistic activity with other defense systems or the involvement of accessory genes are likely to more accurately represent bacterial defense (Wu et al., 2024), and such information would help to optimise strains used in biotechnology. Lack of knowledge of the general defense landscape could cause unfavourable deletions in the genomic engineering process, undermining the defensive function. Metabolic models benefit from regulatory information to make predictions for mutant strain viability, especially by settling differences between experimental observations and simulation results (Puchałka et al., 2008). The systems-approach to bacterial immunity could be key in adding reliability to strains used in biotechnology.

The predicted defense function of PD-T7-1 and RMII in *P. putida* PaW85 remains to be verified with novel phages, isolated with strains lacking the systems. The experimental validation of HerA/DUF4297 supports its predicted role and directs future research towards regulation mechanisms specific to *P. putida.* All these systems could be investigated as part of the orchestrated protection against phage infection. Since interactions with phages require frequent evolution, bacterial immunity is more complex than disparate parts providing cumulative effect (Wu et al., 2024). Thus, verification of the widely available prediction tool outputs of likely candidates, combined with functional studies, could improve the understanding of bacterial defense, increasing the capacity of existing and novel biotechnology applications.

SUMMARY

Phages are a constant threat to bacterial survival, causing bacteria to evolve anti-phage defense systems, which protect from infection. While *P. putida* is highly relevant in biotechnology, its defense systems are understudied. Optimising strains through genomic deletions is often a necessary step in enhancing or providing new abilities to bacteria, and the quality improves with understanding the organism better. The development of bioinformatic prediction tools has supported research in this area, expanding the scope of bacterial defense and setting the basis to further characterise the systems composing it. This thesis aimed to verify predicted anti-phage defense systems PD-T7-1, RMII and HerA/DUF4297, in *P. putida* PaW85. By constructing individual deletion mutants of the systems, their impact on susceptibility to phages from the CEPEST *P. putida* phage collection could be tested. Although the defensive role of PD-T7-1 and RMII could not be confirmed due to inconsistent changes in the susceptibility of the mutants to phages, there are still indications of potential functionality. This sets forth research directions for validating these systems, as the CEPEST collection continues to grow. Increases in phage infection efficiency were observed with the HerA/DUF4297 mutant. This led to complementation experiments, which verified the cause to be loss of function, instead of unintended mutations during the construction process. Further, complementation with an active site mutation of DUF4297 showed that this protein likely functions as a nuclease. The verification of HerA/DUF4297 as an anti-phage defense system in *P. putida* sets a precedent to further characterise the activation mechanisms of the complex. The studied enzymes contain shared domains with previously identified anti-phage defense systems, however, there is variability which points to likely distinct activation mechanisms. This highlights the mosaic nature of bacterial defense systems, which are adaptive and uniquely tailored to each organism. Verifying predicted anti-phage defense systems in *P. putida* could improve genomic engineering, resulting in more reliable applications. Continuous progress in novel phage isolation and bacterial anti-phage defense research will help to utilise the potential of microbial genetics, with implications in shifting energy to renewable sources, sustainable production methods and agricultural development.

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