

BRIEF REPORT

ENVIRONMENTAL MICROBIOLOGY



Isolation and characterization of a phage collection against *Pseudomonas putida*

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Abstract

The environmental bacterium, *Pseudomonas putida*, possesses a broad spectrum of metabolic pathways. This makes it highly promising for use in biotechnological production as a cell factory, as well as in bioremediation strategies to degrade various aromatic pollutants. For *P. putida* to flourish in its environment, it must withstand the continuous threats posed by bacteriophages. Interestingly, until now, only a handful of phages have been isolated for the commonly used laboratory strain, *P. putida* KT2440, and no phage defence mechanisms have been characterized. In this study, we present a new Collection of Environmental *P. putida* Phages from Estonia, or CEPEST. This collection comprises 67 double-stranded DNA phages, which belong to 22 phage species and 9 phage genera. Our findings reveal that most phages in the CEPEST collection are more infectious at lower temperatures, have a narrow host range, and require an intact lipopolysaccharide for *P. putida* infection. Furthermore, we show that cryptic prophages present in the *P. putida* chromosome provide strong protection against the infection of many phages. However, the chromosomal toxin–antitoxin systems do not play a role in the phage defence of *P. putida*. This research provides valuable insights into the interactions between *P. putida* and bacteriophages, which could have significant implications for biotechnological and environmental applications.

INTRODUCTION

Pseudomonas putida, especially the strain KT2440 and its isogenic PaW85, has long served as a model species of environmental bacteria. Its main features include high stress tolerance and a wide variety of different metabolic pathways (Dos Santos et al., 2004; Nikel et al., 2016; Reva et al., 2006). The versatile metabolism of *P. putida* has increased the interest of the scientific and biotechnology communities in utilizing

this microbe in bioproduction and biodegradation applications. To engineer a robust microbial cell factory from this laboratory workhorse, the genome of *P. putida* has been significantly edited and reduced (Aparicio et al., 2019; Martínez-García et al., 2014; Martínez-García & De Lorenzo, 2024). Notably, while *P. putida* is considered to have high potential for bio-industry, it is curious that there is no knowledge regarding how this bacterium protects itself from bacteriophages.

Phages are abundant in the environment with the number of phage virions on Earth estimated to reach up to 10^{31} (Comeau et al., 2008). The variety of phages

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is vast and includes DNA and RNA viruses with double- or single-stranded genomes that can be segmented or unsegmented (Dion et al., 2020). Temperate phages can undergo a lysogenic cycle, enter bacterial genomes as prophages, reproduce at the rate of cell division, and, in certain circumstances, exit the genome and enter the lytic cycle. Lytic phages are unable to enter the bacterial genome. Instead, after their genome is inserted into the bacterium, they reprogram the cell metabolism to produce viruses and finally lyse the exhausted host bacterium to release the new progeny of bacteriophages. Phages pose a constant threat to both basic microbiology studies as well as bacteria-based bio-industrial applications (Bogosian, 2005; Los, 2012). Yet, despite their wide range and variety, only a few phages able to infect the biotechnologically relevant *P. putida* KT2440 or PaW85 strain have been isolated recently (Jaryenneh et al., 2023; Ngiam et al., 2022).

Bacteria are endowed with different mechanisms that protect them against viruses. A vast number of phage defence systems operating in various ways have been revealed to the scientific community in recent years (Doron et al., 2018; Georjon & Bernheim, 2023; Millman et al., 2022; Vassallo et al., 2022). These systems are often encoded outside the core genomes of bacteria and tend to cluster in the so-called defence islands of mobile DNA regions (Makarova et al., 2011). For example, many phage defence systems can be found in prophages, that is, in genomes of temperate phages that have been integrated into the bacterial chromosome (Bondy-Denomy et al., 2016; Patel & Maxwell, 2023). Among the widespread anti-phage mechanisms are restriction-modification systems (Tock & Dryden, 2005), CRISPR-Cas modules (Horvath & Barrangou, 2010), and various types of abortive infection systems (Abi systems) (Lopatina et al., 2020). Some Abi systems have been shown to act through a toxin–antitoxin (TA) mechanism (Bouchard et al., 2002; Dy et al., 2014; Fineran et al., 2009).

TA systems are widespread bacterial genetic elements that code for a potentially harmful toxin protein and an antidote, an RNA or a protein, able to inhibit the toxicity of its partner efficiently. TA systems are known to stabilize plasmids and other mobile elements (Fraikin & Van Melder, 2024; Ogura & Hiraga, 1983; Wozniak & Waldor, 2009), but besides that, they have also been reported as defence systems against phages (Kelly et al., 2023; LeRoux & Laub, 2022). Phage infection has been shown to activate toxins. This, in turn, triggers the cell death or severe growth inhibition of phage-infected bacteria (Dy et al., 2014; Fineran et al., 2009; Hazan & Engelberg-Kulka, 2004; LeRoux et al., 2022), which limits the phage propagation within the bacterial population. Upon activation, some toxins may even preferentially target phage products or

processes (Guegler & Laub, 2021). Toxins can be activated either by phage-caused overall shutoff of host transcription, followed by destabilization of the RNA-protein TA complex (Guegler & Laub, 2021; Short et al., 2018) or by direct binding of a phage structural protein resulting in toxin liberation (Zhang et al., 2022). Still, despite accumulating evidence of TA systems acting as anti-phage elements, in most cases, the participation of TA systems in phage defence has not been confirmed. Moreover, the activation mechanism of the toxin is often unidentified.

P. putida PaW85, as well as its isogenic KT2440 strain, harbours numerous chromosomal TA systems. Deletion of as many as 13 TA systems from the *P. putida* PaW85 genome (strain Δ 13TA) revealed that multiple TA loci confer no clear fitness benefits but rather impose slight fitness costs to the bacterium, given that the presence of TA loci decreased the competitive fitness of wild-type *P. putida* (Rosendahl et al., 2020). Yet, so far, we have tested the effects of the lack of chromosomal TA loci on stress tolerance, persistence, biofilm formation, and competitive fitness (Rosendahl et al., 2020), but we have not yet analysed whether the TA systems can protect *P. putida* from invading phages. As phage defence has been considered to be an important function of TA systems (Kelly et al., 2023; LeRoux & Laub, 2022; Song & Wood, 2020), it is reasonable to test the possibility that TA loci, despite their cost, are maintained in the *P. putida* PaW85 chromosome due to their importance in phage attacks.

Here, we present a collection of environmental bacteriophages isolated with a *P. putida* PaW85 derivative lacking 13 chromosomal TA loci and 4 cryptic prophages. Twenty-two phage species from nine genera were characterised by their genome sequences, taxonomy, morphology, temperature requirements, and host and receptor specificities. This first library of *P. putida* phages allowed us to test whether TA systems or prophages could be involved in the phage defence of *P. putida*. The collection opens up many research directions to follow, as the need to catch up on phage defence research of this bacterium is of crucial importance to the scientific and bio-industry communities.

EXPERIMENTAL PROCEDURES

Bacterial strains, media, and growth conditions

The bacterial strains and plasmids used are listed in Table S1 (Supplementary File 1). All strains constructed in this study are derivatives of *P. putida* PaW85 (Bayley et al., 1977), which is isogenic to well-studied KT2440 (Regenhardt et al., 2002). Bacteria were grown in lysogeny broth (LB). If selection was



necessary, the growth medium was supplemented with kanamycin ($50 \mu\text{g ml}^{-1}$) for *Escherichia coli* and benzylpenicillin ($1500 \mu\text{g ml}^{-1}$), kanamycin ($50 \mu\text{g ml}^{-1}$) or streptomycin ($200 \mu\text{g ml}^{-1}$) for *P. putida*. *E. coli* was incubated at 37°C and *P. putida* at 30°C , except for phage infection experiments that mainly were conducted at 20°C . Bacteria were electrotransformed according to the protocol of Sharma and Schimke (1996).

Construction of strains

P. putida $\Delta 4\phi$ and $\Delta 13\text{TA}\Delta 4\phi$ strains were constructed by sequential deletion of four prophages from *P. putida* wild-type PaW85 and its $\Delta 13\text{TA}$ derivative. The pEMG- and pSNW2-based plasmids were used for strain construction according to a well-described protocol (Martínez-García & de Lorenzo, 2011). Plasmids are listed in Table S1 (Supplementary File 1), and oligonucleotides used in PCR amplifications are listed in Table S2 (Supplementary File 1). Notably, first, the ΔP1 and the $\Delta 13\text{TA}\Delta \text{P1}$ strains devoid of prophage P1 were obtained by prophage spontaneous deletion when we aimed to delete the P1-encoded *hicAB2* (PP_3900-3899) TA system by using plasmid pEMG- ΔhicAB2 . However, instead of the *hicAB2* locus deletion, the whole P1 excised from the *P. putida* genome. The prophage P1 genome is surrounded by 67-bp-long direct repeats *attL* (genomic location 4371608-4371674) and *attR* (4427499-4427565) at each end, and sequencing of ΔP1 and the $\Delta 13\text{TA}\Delta \text{P1}$ strains revealed that recombination between *attL* and *attR* had created a clean P1 deletion leaving one *att* site. *P. putida* ΔP1 and $\Delta 13\text{TA}\Delta \text{P1}$ strains were used to delete other prophage genomes in the order P4, P3, and P2. The pEMG-based plasmids containing prophage deletion loci that were employed for strain generation were a generous gift from Martínez-García et al. (2015). For the deletion of *wbpL* (PP_1804) gene, the upstream and downstream regions of *wbpL* were amplified separately with primer pairs del1804Eco/del1804 and del1804-pikk/del1804Bam, respectively. Two PCR products were joined into an approximately 1-kb fragment by overlap extension PCR using primer pair del1804Eco/del1804Bam, and inserted into EcoRI-BamHI opened plasmid pSNW2 (Volke et al., 2020), a *gfp*-containing derivative of pEMG.

Isolation of bacteriophages

The enrichment method was used to isolate phages from various soil and water samples. The sampling date and exact source for each phage are indicated in Table S3 (Supplementary File 2). *P. putida* PaW85 $\Delta 13\text{TA}\Delta 4\phi$ strain was used as the host bacterium in

the isolation process. To enrich the environmental samples with phages, 5 ml of $10\times$ LB medium, 2 ml of exponential phase ($\text{OD}_{580}\sim 1$) $\Delta 13\text{TA}\Delta 4\phi$ culture, CaCl_2 (final concentration 10 mM), and ciprofloxacin (final concentration $0.01 \mu\text{g/ml}$) were added to the 100 ml of environmental samples. Samples were incubated in flasks overnight at 70 rpm at 20°C . The next day, to get rid of the bacteria and soil sediments, samples were centrifuged for 30 min at 2400 g, and the supernatant was filtrated ($0.22 \mu\text{m}$ filter). To see if the phage isolation was successful, 1 ml of the filtrate was mixed with 200 μl of exponentially growing $\Delta 13\text{TA}\Delta 4\phi$ culture, and 5 ml of 0.3% (w/v) melted LB agar (42°C) containing 10 mM of CaCl_2 and overlaid on LB plates supplemented with ciprofloxacin ($0.03 \mu\text{g/ml}$). Plates were incubated overnight at 20°C . If plaques had formed, single plaques were isolated and purified. To do that, a single plaque was picked and suspended in 100 μl of SM buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 8 mM MgSO_4 , 0.01% (w/v) gelatin). Then, 5 μl of chloroform was added to lyse the bacteria. Ten-fold dilutions of the phage suspension were made, and 2 μl drops were spotted on bacterial lawn plates. Plates were incubated overnight at 20°C . Next, a new single plaque was picked, and the purification step was repeated three times in total. Purified phages were stored in phage storage buffer (SM buffer) both at 4 and -80°C .

Phage DNA purification

For phage DNA extraction, 1–2 ml of phage solution with a titre ranging from 10^8 to 10^{10} PFU/ml was treated with 2 units/ml of DNase and 100 $\mu\text{g/ml}$ RNase (Thermo Scientific), followed by the phage DNA extraction with Phage DNA Isolation Kit (Norgen Biotek) according to the protocol supplied by the manufacturer.

In some cases, the DNA yield from the kit was extremely low, and other measures had to be taken. For the jumbo phages of cluster G3, GeneJET Genomic DNA Purification Kit columns (Thermo Scientific) proved more efficient. The protocol of the phage DNA purification (Norgen Biotek) kit was followed until DNA binding when columns from the genome extraction kit (Thermo Scientific) were used instead of the phage kit columns for binding and elution of the DNA. In some other cases when the DNA yield was low (e.g., phages from genus clusters G6, G7, and G9), the phages were first precipitated from phage lysate with 80 mM ZnCl_2 for 5 min at 37°C , pelleted by centrifugation at 10,000 g for 1 min and then resuspended in 400 μl of TES buffer (0.1 M Tris pH 8.2, 0.1 M EDTA, 0.3% [w/v] SDS). Phage capsids were digested by adding 4 μl of proteinase K (Thermo Scientific) and incubating for 1 h at 60°C , after which the proteins were precipitated with 40 μl 3 M K-acetate (pH 4.8) on ice for



15 min. The pelleted proteins were removed by centrifugation (1 min, 10,000 g at 4°C) and DNA in the supernatant was pelleted by adding a 1:1 volume of isopropanol to the liquid fraction and incubated on ice for 5 min. The pellet of DNA was centrifuged down (10,000 g for 10 min), washed twice with 75% (v/v) ethanol, and resuspended in 60–70 µl of water. To purify the precipitated DNA sample, it was cleaned with a DNA Clean-up and Concentration kit (Zymo Research) according to the manufacturer's protocol and eluted with 35 µl of water. Regardless of the method used, all the purified phage DNA samples were sequenced.

Sequencing and assembly

Whole genome sequencing was done with Illumina MiSeq PE 2 × 175 bp or PE 2 × 151 bp setup. The average sequencing depth was 120×. Sequencing reads were filtered with fastp v.0.21.0 (Chen et al., 2018) and assembled with SPAdes v.3.15.4 using isolate mode (Priibelski et al., 2020). Genome completeness was confirmed by checking the existence of technical repeats in both contig ends with an exact length of the largest k-mer used during the assembly process by SPAdes and one repeat copy was removed.

Genome annotation and characterization

tRNA and protein-coding genes were annotated using Prokka v.1.14.6 (Seemann, 2014) together with PHROGs (Terzian et al., 2021). Overlaps between ORFs and tRNAs were allowed. Average GC% and coding potential were calculated for each genome. Assembled contigs were reordered for easier visualization of genomic comparisons. Phages with a close relative already available in the databases were reordered similarly to the published genome. For new species and genera, we used PhageTerm v.1.0.12 (Garneau et al., 2017) to predict packaging strategy and termini. If sequencing depth and library method allowed reliable PhageTerm prediction, the same predicted termini were used for other same species phages (identity >95%) as well. Genomes without reliable PhageTerm prediction in any of the close relatives were reordered by setting a large terminase subunit as the first gene if there was no overlap with the preceding ORF. In case of overlaps, arbitrary non-intragenic start was chosen but a similar start was ensured in the isolates belonging to the same species or in the same genus if possible.

Phage lifestyle prediction was carried out with PhageBOX software (Shang et al., 2023) and additional independent recombinase and integrase prediction using HMMER (<http://hmmer.org/>) and Pfam models PF00589, PF00665, PF07508, PF13009, and PF16795. Possible homology to *P. putida* genes

including prophage regions was checked with phages' protein and gene sequences as well as whole genomes using BLAST (Altschul, 1997; Altschul et al., 1990; Zhang et al., 2000).

Genomes were submitted to GenBank under BioProject PRJNA1067406, the accession numbers are shown in Table S3 (Supplementary File 2).

Taxonomical clustering analyses

For the comparison to available published phage genomes, we used the monthly updated INPHARED database (Cook et al., 2021) (version date 1 February 2024) including ~27,000 complete or near complete phage genomes available in GenBank. To limit the number of genomes for further similarity analysis, vCONTACT was used (Bin Jang et al., 2019) to select all possible close relatives from Inphared that cluster together with *P. putida* phages in the sequenced library. vCONTACT was run with settings —rel-mode 'Diamond' —pcs-mode MCL —vcs-mode ClusterONE on a dataset consisting of all the protein sequences from Inphared and 67 library phages. vCONTACT picked out 102 phages from Inphared that were grouped with one or more library phages. The genome sequences of all 102 phages were then added to 67 library phages for VIRIDIC analysis to find genome-wide similarities and determine more specific species and genera classification as much as possible (based on agreed thresholds of genome sequence identity over 95 and 70% correspondingly; Turner et al., 2021). In the case of borderline species identity (close to 95%), further detailed comparisons regarding the gene content and locations were made using Mauve (Darling et al., 2004) to align and visualize the annotated genomes. Whole genome coding sequence comparisons were visualized with clinker (Gilchrist & Chooi, 2021).

Phylogenetic tree

Phylogenetic trees were based on the muscle (Edgar, 2004) alignment of four concatenated protein (22 representative species) or gene sequences (all 67 phages): major head protein, DNA primase, spanin, and terminase large subunit. Trees were calculated with iqtree v.1.6.12 (Nguyen et al., 2015) using ModelFinder (Kalyaanamoorthy et al., 2017) and 1000 bootstrap replicates and visualized in iTOL web application (Letunic & Bork, 2021).

Transmission electron microscopy

For morphology analysis by transmission electron microscopy (TEM), the phage samples were concentrated by polyethylene glycol (PEG) precipitation. For



that, 1 ml of phage filtrate ($\sim 10^9$ PFU/ml) was treated with 5.3% (w/v) PEG 8000 and 0.33 M NaCl. After 1.5 h incubation at 4°C, the phage particles were pelleted at 8000 g for 10 min at 4°C. The phage precipitate was allowed to resuspend slowly in 20 μ l SM buffer overnight at 4°C. Then, 10 μ l of high-titre phage suspension ($\sim 10^{11}$ PFU/ml) was incubated for 5 min on a formvar/carbon-coated grid. After the excess liquid was removed with filter paper, negative staining of phage samples was performed for 2–3 min with an aqueous solution of 2% (w/v) phosphotungstic acid or 2% (w/v) uranyl acetate. Excess liquid was removed, and the grids were rinsed in demineralized water and air-dried. The TEM images were taken using a Tecnai G2 Spirit BioTwin TEM at a 120 kV accelerating voltage, and images were captured using an Orius SC1000 camera.

Plaque assays

Bacteria were grown in LB medium overnight at 20°C. The bacterial cultures were diluted 15-fold into fresh LB medium and grown until $OD_{580} \sim 1$ at 20°C. Next, 200 μ l of the bacterial culture was mixed with 5 ml of melted 0.3% (w/v) LB agar medium (42°C) containing 10 mM $CaCl_2$ and overlaid on 1.5% (w/v) LB agar plates containing 0.03 μ g/ml or 0.01 μ g/ml ciprofloxacin to create a bacterial lawn. For qualitative phage resistance assay, 1.5 μ l drops of phage lysates with the maximum titre were spotted on the bacterial lawn plates. Plates were incubated overnight at 20°C. The formation of plaques was assessed.

To quantify the infectivity of phages, phage lysates were standardized to a titre of 10^7 – 10^8 PFU/ml. Then, 1.5 μ l drops of ten-fold dilutions of phage lysates were spotted on the bacterial lawns. Plates were incubated overnight at 20°C (if not mentioned otherwise). The number of plaques formed was counted to calculate the efficiency of plating in plaque-forming units per millilitre.

RESULTS

Phage isolation and genome sequencing

To address the question of the importance of chromosomal TA systems in *P. putida* PaW85 phage defence, we started with isolating phages from different environmental samples, mainly muddy water and different types of soil. To rule out the possibility of TA systems protecting the cells from phage infection and thus inhibiting phage isolation, we decided to use the *P. putida* PaW85 strain lacking 13 genomic TA systems (Rosendahl et al., 2020). Also, to isolate a diverse phage collection of *P. putida* phages, we needed as sensitive a *P. putida* derivative as possible. While nothing was previously known about the phage resistance

of *P. putida* PaW85, it is commonly well-established that genomic prophages often carry phage-defence elements (Bondy-Denomy et al., 2016; Dedrick et al., 2017; Patel & Maxwell, 2023). *P. putida* PaW85 genome harbours four cryptic prophages (Martínez-García et al., 2015), and presuming that they may contribute to phage resistance, we deleted all these prophages from the strain that already lacked 13 TA systems. Thus, as a host for phage isolation, we used *P. putida* strain $\Delta 13TA\Delta 4\phi$ (Figure 1A).

Our first isolation attempts at the optimal growth temperature for *P. putida* of 30°C were unsuccessful. Thus, we optimized the phage isolation protocol by lowering the incubation temperature. This was motivated by the fact that *P. putida* is an environmental bacterium that usually lives in the soil and water at much lower temperatures than 30°C. As expected, reducing the temperature of the phage isolation procedure to 20°C was fruitful and allowed us to collect 67 phages from different environmental samples (Table S3, Supplementary File 2; see Figure 1B for a scheme of phage isolation process). The phage collection was named CEPEST from the Collection of Environmental *P. putida* Phages from Estonia.

All isolated phages were sequenced, successfully assembled into complete genome sequences, and annotated. VIRIDIC (Moraru et al., 2020) clustering analysis based on sequence similarity of assembled genomes demonstrated that 67 isolated phages belong to nine different genus clusters G1–G9 (Figure 2A; Figure S1, Supplementary File 3). Most sequenced genomes were 39–42 kb long, which is the most common length range among published phages (Figure S2, Supplementary File 3), and contained 45–52 open reading frames and no tRNA genes. Two genus clusters differed regarding the genome size. Cluster G1 phages with genome sizes 95–97 kb belonged to the size class that has so far been poorly represented among sequenced phages (Figure S2, Supplementary File 3). G1 genomes contained 169–176 protein-coding sequences and 16–17 tRNAs. Cluster G3 included eight phages with a genome length over 200 kb and were thus defined as jumbo phages.

All 67 phages appeared to have a lytic lifestyle as no recombinase or integrase sequences were detected in their genomes. Only cluster G3 phages contain UvsX-like recombinase. UvsX orthologs have been previously reported to be present in phages with large genomes that have strictly virulent lifestyles (Lopes et al., 2010).

Genomic GC content varied from 47.2 to 61.5%, being the lowest in G1 phages and highest in a G8 phage. All informative statistics for each sequenced genome are presented in Table S3 (Supplementary File 2).

Phage phylogeny, taxonomy, and diversity

We compared all 67 sequenced genomes to complete phage genomes in GenBank provided in the Inphared

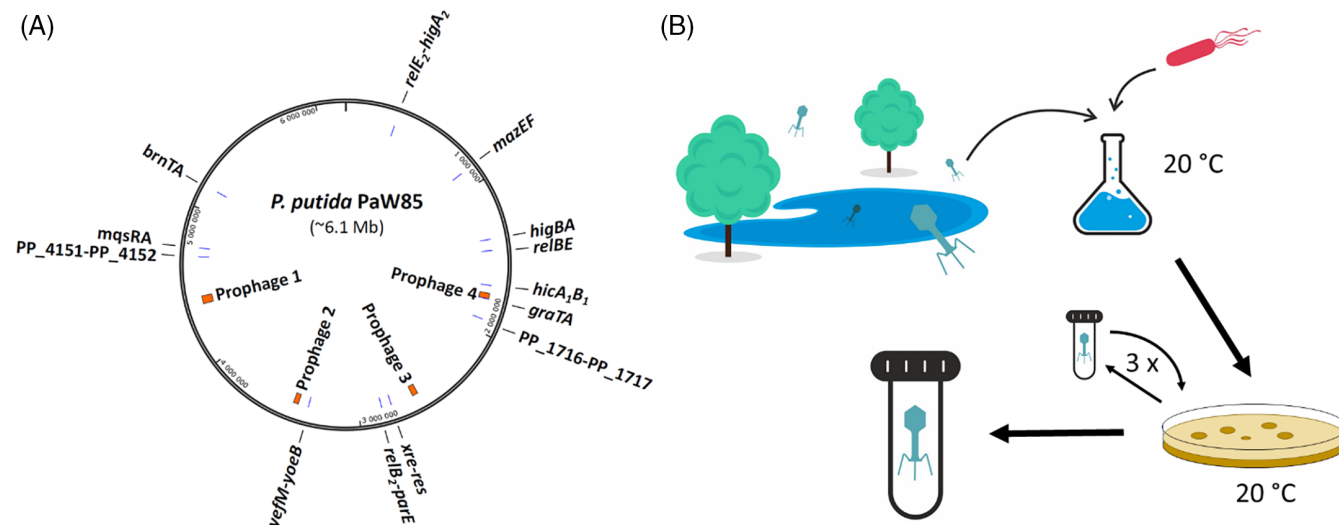


FIGURE 1 Phage isolation process by using *Pseudomonas putida* devoid of 13 TA systems and four prophages. (A) Schematic representation of *P. putida* PaW85 genome. The location of 13 TA systems and 4 prophages that are deleted from the phage isolation strain *P. putida* Δ13TAΔ4φ are indicated. The figure was made with SnapGene software (www.snapgene.com). (B) A scheme of phage isolation process: environmental samples were incubated with the host *P. putida* Δ13TAΔ4φ overnight at 20°C. The culture was then cleared and overlay agar plates poured to visualize phage plaques. Individual plaques were purified three times before making a final phage monoculture stock.

collection (Cook et al., 2021) as of 1 February 2024, to determine taxonomical affiliation and distribution. We clustered proteins in CEPEST phages with all the proteins in Inphared collection phages using the vCONTACT taxonomic clustering tool to detect possible close relatives among already published complete phage genomes. Inphared phages that grouped with at least one of the CEPEST phages were included in the following inter-genomic similarity analysis using VIRIDIC. Based on the agreed 70% genus level identity threshold in phage taxonomy (Turner et al., 2021), the sequenced library revealed nine separate genus clusters. Most of the published phages that were included appeared to have a genomic similarity of less than 70% to library phages. However, six of the nine clusters contained one or more genomes from Inphared that exceeded either genus or even species-level similarity threshold. This enabled us to classify phages in cluster G2 as members of the genus *Pifdecavirus*; phages in G4, G7, and G9 as members of the genus *Ghunavirus*; and phages in G6 belonged to the genus *Pollyceevirus* (Tables S7, S10, and S12, Supplementary File 2). A phage belonging to cluster G8 had a low genus level similarity (~72%) to two *P. putida* phages LNA8 and LNA10 which are currently taxonomically unclassified (Ngiam et al., 2022) (Table S11, Supplementary File 2). Phages in three genus clusters (G1, G3, and G5) did not reach genus-level similarity (70%) to any of the available phage genomes, suggesting three new genera of phages (Tables S4–S6 and S8, Supplementary File 2).

Only two phages belonging to cluster G4 (*Ghunavirus*) had species-level relatives among the Inphared

collection. Phage Emajogi showed >95% similarity to *Pseudomonas* phages CHF7 (MN729596), phiPSA2 (KJ507099), and phiPsa17 (KR091952). Phage Luke-2 had >96% similarity to *Pseudomonas* phage Pst_GIL1 (OK523999) and >95% similarity to *Pseudomonas* phage KNP (KY798121) (Figure S3A, Supplementary File 3; Table S7, Supplementary File 2).

Jumbo phage cluster G3 contained one phage named Aura with a genome size just below the jumbo phage length threshold (199,504 bp) but showed high similarity on genome sequence level and was therefore assigned to species cluster 3A together with Illi-2 and SKa-4 jumbo phages (Table S6, Supplementary File 2).

Genus clusters G2 and G5 were the most abundantly represented in our collection (Figure S1, Supplementary File 3). The majority of the isolates in both of those clusters were assigned to one species based on a comparison of genome sequences, but the third new genus cluster, G5, needed additional analysis to decide the species assignment. There seemed to be three separate species in G5, but the final division remained ambiguous for two phages—SKa-3 and Kompost-1. VIRIDIC automatic clustering grouped these phages with phage BotAed (species cluster 5C). However, the similarity matrix showed SKa-3 and Kompost-1 being more closely related to phages in species cluster 5B (Table S8, Supplementary File 2). In addition, BotAed contains a gene for cysteine dioxygenase, which is not present in other G5 phages, including SKa-3 and Kompost-1 (Figure S3B, Supplementary File 3). Therefore, SKa-3 and Kompost-1 were marked as species 5B* (Table S8, Supplementary File 2).

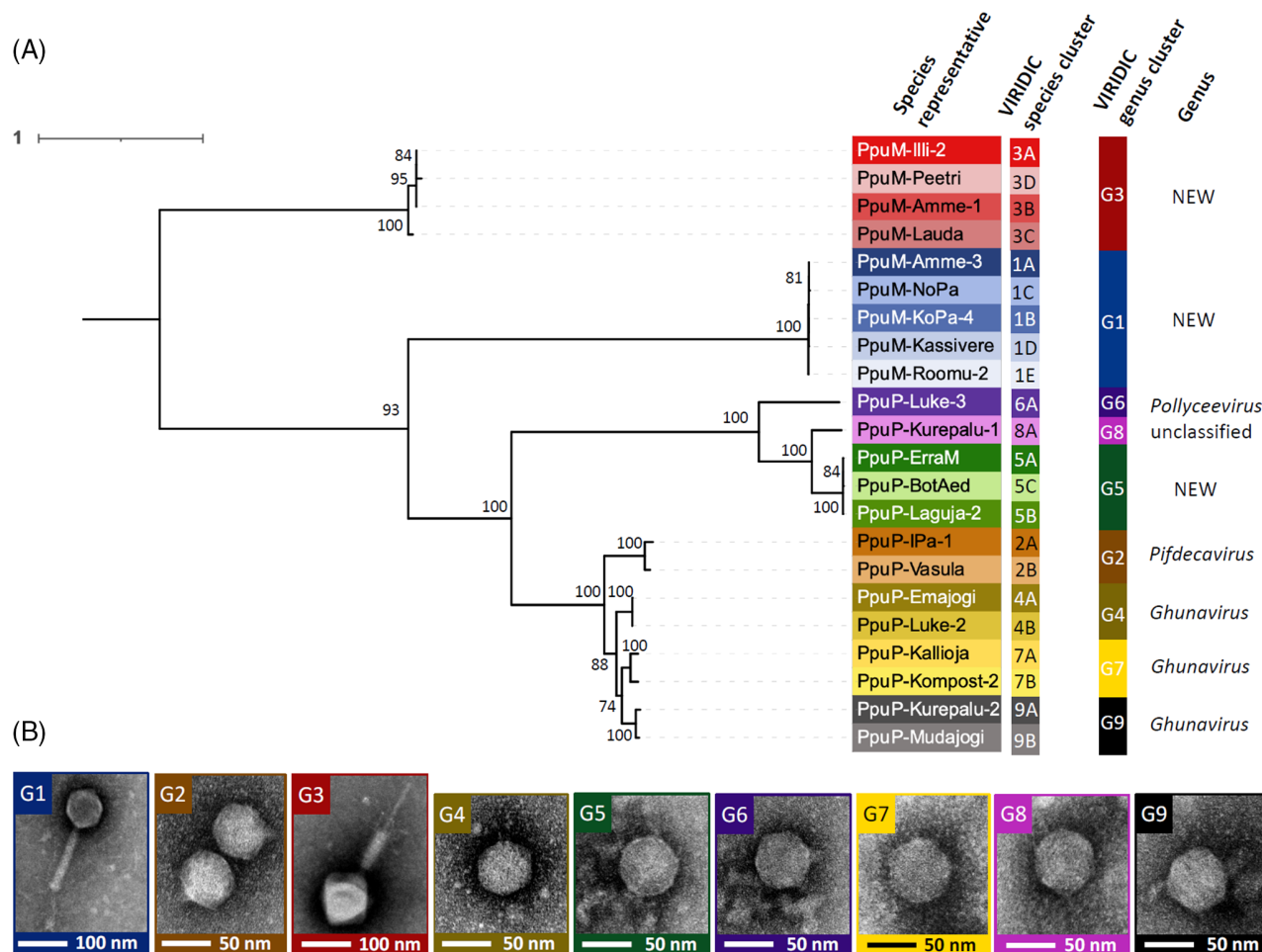


FIGURE 2 The phylogenetic and morphological analysis of phages in CEPEST collection. (A) Midpoint-rooted phylogenetic tree of 22 representative phage species from 9 clusters (G1–G9). The tree is based on multiple alignment of four concatenated protein sequences (major head protein, DNA primase, spanin, and terminase large subunit), calculated with IQ-TREE using ModelFinder and visualized using iTOL online tool. Bootstrap values >70 are shown. Dark colours mark different VIRIDIC genus clusters, and lighter tones of each colour show species belonging to the corresponding clusters. (B) Morphology of phages in CEPEST collection. A negative staining TEM image of a representative phage from each genus cluster in the CEPEST collection (G1—NoPa, G2—Vasula, G3—Amme-1, G4—Emajogi, G5—Laguja-2, G6—Luke-3, G7—Kompost-2, G8—Kurepalu-1 and G9—Kurepalu-2).

Gene content analysis was also made for phage Vanda in G5. Although Vanda had genomic similarity reaching over 95% species level threshold with some 5B phages (Table S8, Supplementary File 2), we still marked Vanda as a 5A species phage. Namely, Vanda lacked a protein kinase coding gene similar to 5A phages ErraM (Figure S3B, Supplementary File 3) and ErraS in contrast to 5B or 5C phages. Vanda belonging to 5A was also supported by VIRIDIC automatic species clustering.

In conclusion, VIRIDIC clustering, sequence similarity matrix, gene content, and organization comparison resulted in 22 representative species among 9 identified genus clusters (Figure 2A). Twenty of those were new species without close and characterized relatives among publicly available bacteriophage genomes. Therefore, to know more about those phages, we set

out to determine the morphology of the isolated phages with TEM. At least one representative species from each of the nine phage genera was analysed. TEM results demonstrated that most of the phages in our collection had podovirus morphology, and only phages from genus clusters G1 and G3 represented myoviruses (Figure 2B; Table S3, Supplementary File 2). All the phages with podovirus virions share a similar genome and capsid size, around 40 kbp and 50–60 nm in diameter, respectively. Myoviruses from genus clusters G1 and G3 that have significantly larger genomes (almost 100 kbp for G1 and over 200 kbp for G3) than podoviruses also have larger capsids (Figure 2B). For instance, the jumbo phages from genus cluster G3 have the largest virions of the collection with a head diameter of almost 100 nm. Thus, the morphological studies demonstrate that the genome sizes correlate

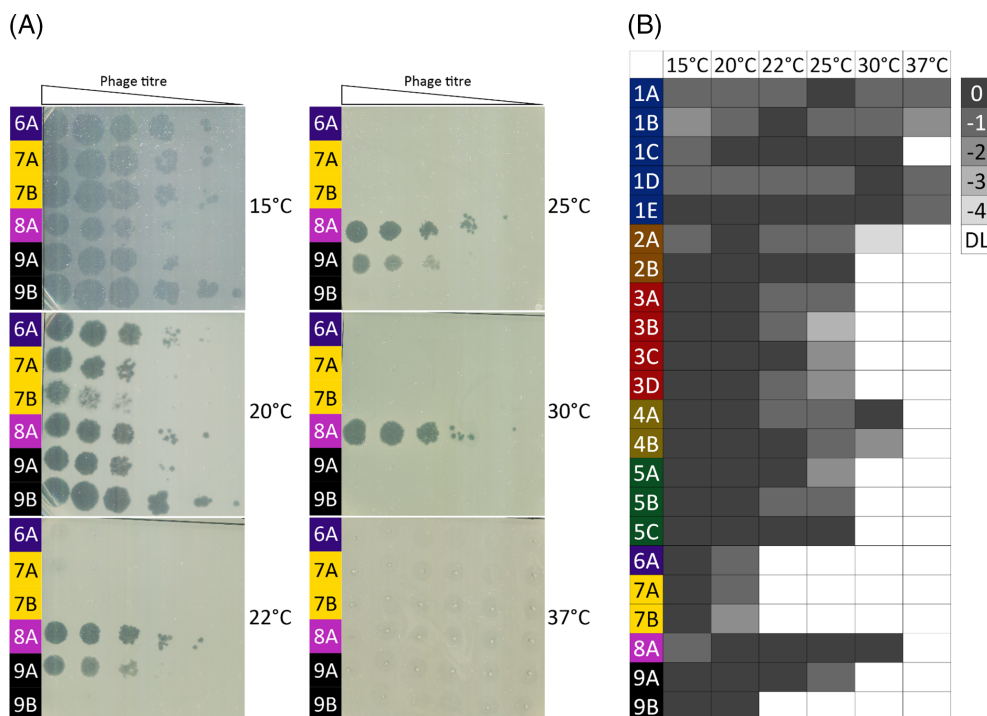


FIGURE 3 Temperature sensitivity of CEPEST phages. The infection efficiency of each phage species representative was tested at temperatures from 15 to 37°C. A: Qualitative infectivity of phages from genus clusters G6, G7, G8, and G9. (B) Heatmap of infection of EOP of each phage at a certain temperature. Zero level shows the most efficient infection, and numbers (and decreasing intensity of grey colour) represent a 10-time decrease in EOP. DL (white) is the detection limit of the experiment, no infection could be detected.

with the morphology of the phages, with the two myovirus genera having larger genomes and larger virions than the podovirus phages in our collection.

Temperature-sensitivity of *P. putida* phages

Given that our first attempts for phage sampling at 30°C were unsuccessful and that the phage library was collected at 20°C, we hypothesised that the infection of *P. putida* phages might depend on the temperature. To determine the temperature range and sensitivity of the phages, we measured their infection ability at various temperatures, ranging from 15 to 37°C. The results showed that at 30 and 37°C most of the isolated phages were unable to infect the used *P. putida* host $\Delta 13TA\Delta 4\phi$ (Figure 3). Moreover, the infection efficiency of most phages was already strongly reduced at 25°C, with the phages from genera G6 and G7 and the species 9B losing their infection ability already at 22°C. However, all phages retained the ability to infect the *P. putida* cells at 15°C, and, except for most genera G1 and G8 species, even presented the strongest infection phenotype at this low temperature (LT; Figure 3). The most insensitive to changes in infection temperature were the phages from the genus cluster G1, as they (except for 1C phage NoPa) were infectious at all

tested temperatures. Remarkably, the temperature sensitivity of phages can vary between species in one genus, for example, phage 1C and most strongly 9B losing the ability to infect cells at clearly lower temperatures than other members of the corresponding genera (Figure 3; Figure S4, Supplementary File 3). Taken together, most *P. putida* phages in our collection are temperature-sensitive and lose their infection efficiency at higher than 20–25°C.

Prophages, but not chromosomal TA systems, increase the resistance of *P. putida* against several phages

To address our starting goal of studying the importance of 13 chromosomal TA systems in the phage defence of *P. putida* PaW85, we compared the phage resistance of the *P. putida* wild-type strain PaW85 and its $\Delta 13TA$ derivative. Analysis of the infection of the 22 phages representing each species showed that the absence of TA systems did not affect the infection efficiency of phages (Figure 4A,B). Given that several phage species in our collection contain multiple phage isolates with slightly varying genome identities (Tables S3–S12, Supplementary File 2), we also compared the phage resistance of wild type and $\Delta 13TA$ with other 45 phages in our collection. Similar to the

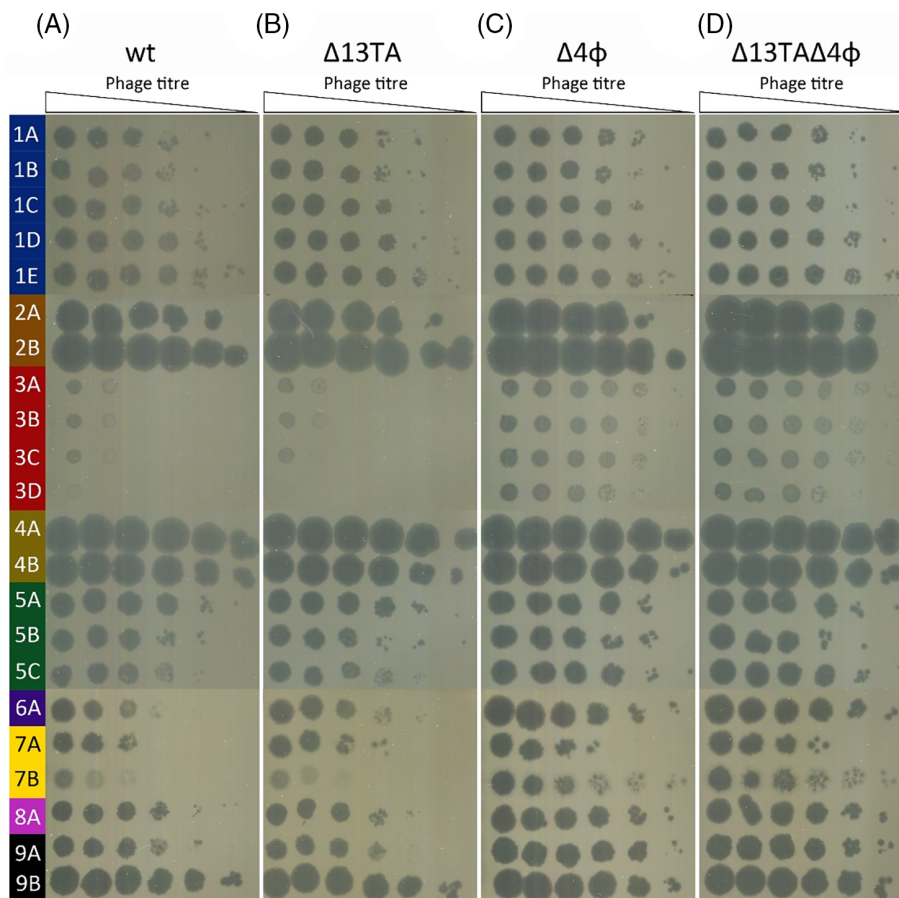


FIGURE 4 *Pseudomonas putida* PaW85 prophages provide protection against several CEPEST collection phages. Quantitative EOP measurement of phage infection on bacterial lawns of (A) *P. putida* PaW85 (wt), (B) *P. putida* PaW85 lacking 13 TA systems ($\Delta 13TA$), (C) *P. putida* PaW85 lacking four prophages ($\Delta 4\phi$), (D) *P. putida* PaW85 lacking 13 TA systems and four prophages ($\Delta 13TA\Delta 4\phi$). Then, 1.5 μ l drops of 10-fold serial dilutions of phages were spotted on the strain to be tested, and plates were incubated overnight at 20°C. EOP was counted from the plaques in dilution spots.

data obtained with 22 representatives of each species (Figure 4A,B), we recorded no difference between the phage sensitivity of the *P. putida* wild-type and $\Delta 13TA$ strains (data not shown). Thus, the 13 chromosomal TA systems of *P. putida* do not function as phage defence modules against any of the phages in our collection.

Interestingly, several phages, particularly jumbo phages from genus G3, showed very poor infection efficiency against both the wild-type and the $\Delta 13TA$ strain (Figure 4A,B). This indicated that prophages that were present in wild type and $\Delta 13TA$ but were deleted from the phage isolation strain $\Delta 13TA\Delta 4\phi$ contribute to the phage resistance of *P. putida*. To test this, we compared the wild-type and the prophage-deficient $\Delta 4\phi$ strains and indeed observed that the latter was more sensitive to many phages (Figure 4A,C). The most prominent protective effect of prophages was observed against the G3 jumbo and 7B phages, as the wild-type strain demonstrated more than 1000-fold higher resistance than the $\Delta 4\phi$ strain

(Figure 4A,C). Prophages also provided clear protection against the phage species 5C, 6A, 8A (about 10-fold), and 9A (about 100-fold). At the same time, infection of phages from genera G1, G2, G4, and the species 5A, 5B, and 9B was very little or not at all affected by the presence of prophages (Figure 4A,C). Also, the comparison of the phage resistance of $\Delta 4\phi$ and $\Delta 13TA\Delta 4\phi$ demonstrated similar phage sensitivity of the two strains (Figure 4C,D), which further confirms that 13 TA systems do not contribute to the phage resistance of *P. putida*. Overall, these results show that while several of the phages are insensitive to the presence of the four cryptic prophages in PaW85, the prophages provide a variable level of defence against about half of the phage species in the CEPEST collection. This protection seems not to rely on the inhibition of superinfection of related phages, as bioinformatic analysis detected no significant sequence-based homology between any of the phage genes and *P. putida* PaW85 prophages or other parts of the bacterial genome.

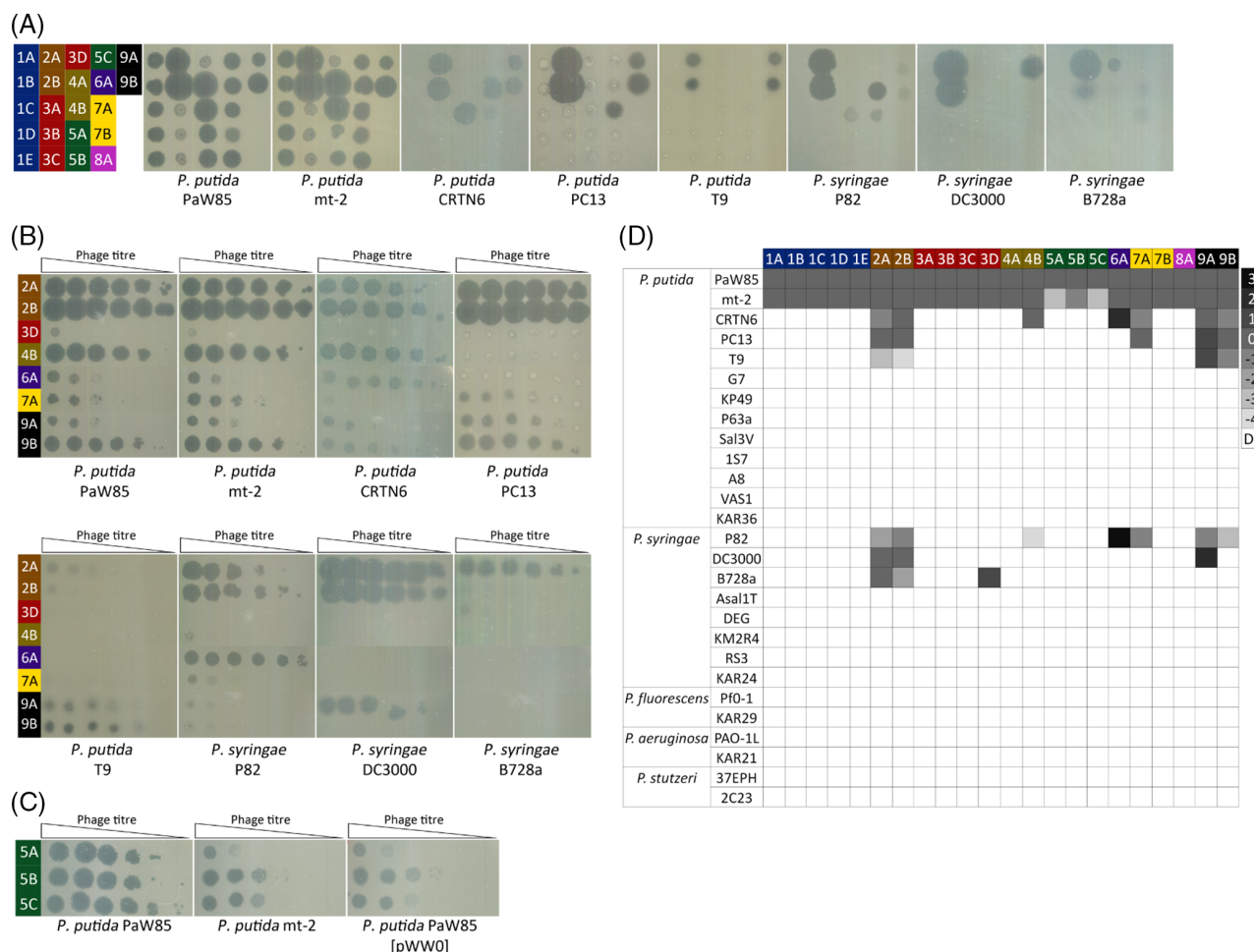


FIGURE 5 Host specificity of CEPEST phages. (A) Qualitative assay of infection of all 22 phage species representatives (indicated in the colour matrix) on different *Pseudomonas* hosts. (B) Quantitative EOP measurement of phage infection on their *Pseudomonas* hosts. Then, 1.5 μ L drops of 10-fold serial dilutions of phages were spotted on the strain to be tested, and EOP was counted from the plaques. (C) EOP of G5 phages on *P. putida* PaW85, its ancestral strain mt-2 containing the pWW0 plasmid, and *P. putida* PaW85 with the pWW0 plasmid. (D) A heatmap of host specificity of phages. Zero level shows the infection of PaW85 reference strain, and numbers (and decreasing or increasing intensity of grey colour) represent 10-fold changes in EOP. DL (white) is the detection limit of the experiment, no infection could be detected. Plates were incubated for 24 h at 20°C.

P. putida phages in the CEPEST collection have a narrow host range

To study the host range of the isolated *P. putida* phages, we tested whether they can infect other bacteria from the genus *Pseudomonas*. To this end, we selected 11 different *P. putida* strains, 8 strains of *P. syringae*, and 2 each of *P. fluorescens*, *P. aeruginosa*, and *P. stutzeri* (Table S1, Supplementary File 1) from the CELMS microbe collection of our institute (http://eemb.ut.ee/celms/main_list.php). As the closest relative to the *P. putida* wild-type strain PaW85, we also included its ancestor strain PaW1 (mt-2) that harbours the TOL plasmid pWW0 (Worsey & Williams, 1975). For the phage susceptibility testing of these 27 *Pseudomonas* strains, we used the highest phage concentrations of all 22 species in our collection (Figure 5A). The experiment was carried out at 20°C, where all tested bacterial strains could grow. The results show that the phages in the

CEPEST collection are highly host-specific, with phages from genera G1, G5, and G8 being unable to infect any other strains apart from *P. putida* PaW85 and its ancestor mt-2 (Figure 5A,D). Only eight phage species out of 22 could infect some *P. putida* (strains CRTN6, PC13, and T9) and *P. syringae* (P82, DC3000, and B728a) strains at 20°C. No phage was able to infect any of the tested *P. fluorescens*, *P. aeruginosa*, or *P. stutzeri* strains (Figure 5A,D).

Out of the eight species of phages that can infect more strains than just the PaW background, the phages of G2 seem to have the widest host range, followed by 9A species. These phages infect not only five of the tested *P. putida* strains but also three (phages from genus G2) or two (phage 9A) *P. syringae* strains. The host range of phage species 3D, 4B, 6A, 7A, and 9B is narrower, but it is interesting to note that the same particular *P. putida* or *P. syringae* strains that were infected by G2 and 9A

phages were also susceptible to some of these phages (Figure 5A,D).

To quantify the infection efficiency of eight phages that could infect different *Pseudomonas* hosts, the phage resistance assay was carried out using serial dilutions of phages (Figure 5B). Results show that phages differ strongly in their ability to infect the tested hosts. Some phages, including 2A, 2B, 4B, 7A, or 9B, lyse some strains equally to *P. putida* PaW85, while they are less efficient against other susceptible hosts (Figure 5B,D). Surprisingly, 3D, 6A, and 9A phages could infect some *P. putida* or *P. syringae* strains even better than the *P. putida* PaW85 reference strain.

The phage sensitivity of *P. putida* PaW85 and its ancestor strain mt-2 seemed highly similar when the strains were infected with high phage titres (Figure 5A). However, infection efficiency quantification experiments revealed that the mt-2 strain was significantly more resistant to genus G5 phages than *P. putida* PaW85 (Figure 5C). The main difference between the two strains is that mt-2 contains the 117 kbp TOL plasmid pWW0. As plasmids have been shown to affect phage infection efficiency (Ngiam et al., 2022), we hypothesised that the TOL plasmid could be behind the protection against G5 phages. We tested a *P. putida* PaW85 that harbours the pWW0 plasmid (lab collection) and, indeed, all of the G5 phages infected this strain 10^2 – 10^4 times less efficiently (Figure 5C), showing that the plasmid pWW0 was causing the reduced infection efficiency of these phages. However, no other phage was affected by the presence of the TOL plasmid.

Interestingly, our results clearly show how two phage species from one genus can have completely different host specificity. Besides having variable infection efficiency towards the same strains, even the strain specificity may differ. For instance, the 4B and 7A

species showed wider specificity: 4B could additionally infect *P. putida* strain CRTN6 and *P. syringae* P82, and 7A could infect *P. putida* PC13. At the same time, the other species in those genera, 4A and 7B, could not infect any cells except the PaW background (Figure 5A). Even more remarkably, phage 9A lysed the *P. syringae* strain DC3000 100 times more efficiently than the reference strain *P. putida* PaW85, while phage 9B could not infect that *P. syringae* strain at all (Figure 5A,B,D). Taken together, 14 out of the 22 species of phages tested can only infect the *P. putida* PaW85 (and its predecessor and derivative strains), showing a very narrow host range for these phages.

Most CEPEST collection phages need intact lipopolysaccharide for *P. putida* infection

As many podo- and myoviruses of Gram-negative bacteria have been shown to use polysaccharide sugar moieties for adsorption (Bertozzi Silva et al., 2016), we set out to test whether the lipopolysaccharide (LPS) of *P. putida* could be the receptor for phage adsorption of *P. putida* phages from our collection. As many phages recognize the outermost O-antigen part of LPS (Lindberg, 1973; Nobrega et al., 2018), we deleted the *wbpL* glycosyltransferase gene that is required for O-antigen synthesis (Rocchetta et al., 1998) from *P. putida* PaW85 wild-type and also from its $\Delta 13TA\Delta 4\phi$ derivative. The latter strain was constructed to test the receptor dependence of the G3 jumbo phages that infect the wild-type bacteria very poorly. We also picked a *P. putida* transposon mutant from our lab strain collection with a disrupted *wbpM* gene. Similarly to WbpL, WbpM is also involved at the beginning of the LPS

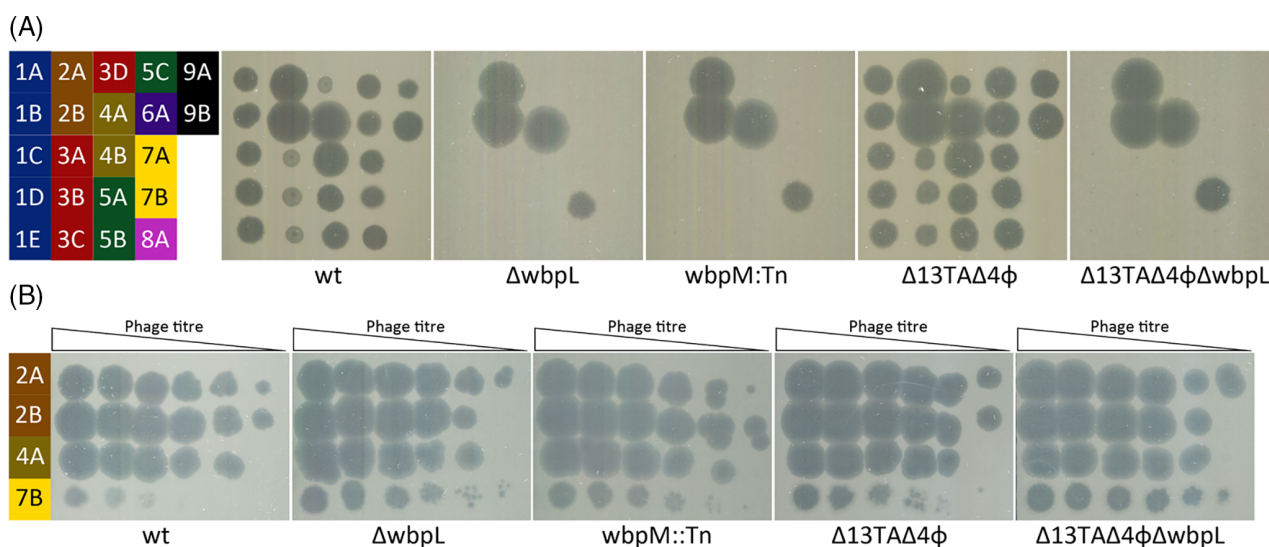


FIGURE 6 Most CEPEST phages require intact LPS for infection. (A) A qualitative assay of phage infection of all 22 phage species representatives on *P. putida* PaW85 wild type (wt) and its *wbpL* or *wbpM* deficient strains and *wbpL* deletion derivative in the $\Delta 13TA\Delta 4\phi$ background. (B) EOP of phages that can infect mutants with deficient LPS. Plates were incubated for 24 h at 20°C before recording the result.



O-antigen synthesis pathway (Creuzenet & Lam, 2001), and its disruption results in LPS molecules deprived of O-antigen moieties (Bélanger et al., 1999). Analysis of these strains showed that out of the 22 phages, 18 could not infect the strains with either the *wbpL* deletion or *wbpM* truncation (Figure 6A). The only phages that retained the ability to infect LPS-deficient *P. putida* derivatives were two species from genus G2 and phages 4A and 7B. These four phages were further analysed to quantify their infection efficiency against O-antigen-deficient strains. Surprisingly, the deficient LPS synthesis significantly increased the infection efficiency of 7B phage Kompost-2, where the $\Delta wbpL$ strain was about 1000-fold more sensitive than the wild-type strain (Figure 6B). For the other three phages (2A, 2B, and 4A), the infection efficiency against the LPS mutants remained unchanged (Figure 6B). These results confirm that the LPS, particularly its outermost O-antigen moiety, is the main receptor for most of the 22 species of phages in our CEPEST collection.

DISCUSSION

The CEPEST collection of bacteriophages isolated during this work represents the first broad selection of *P. putida* environmental phages. Genome homology analysis allocated the CEPEST collection phages into nine genus clusters with 22 species. As only 13 complete *P. putida* phage genome sequences are available to date (February 2024, NCBI database), it is not surprising that many of the phages are entirely new and even constitute several new genus clusters. From that, it seems that extensive studies are still needed to understand the bacteriophage diversity and any new host bacterium used in isolation could result in identifying many new genera of phages.

The number of phage species is variable in the genus clusters of the CEPEST collection

The composition of the CEPEST collection indicates that it has a high representation of some phage species (e.g., 2A and 5B) but is underrepresented in others (e.g., species in genus clusters G6–G9, see Table S3, Supplementary File 2). Currently, the collection contains phages from only nine genus clusters, and the species abundance in different clusters is highly variable. While the genus cluster G8 is represented by only one species, there are also clusters having over ten isolates defined as the same phage species (identity >95%). For instance, 2A and 5B species clusters are the most overrepresented, containing 17 and 21 phage isolates, respectively. Repeated isolation of 2A phages

is not so surprising as G2 phages produce big plaques (Table S3, Supplementary File 2). As plaque sizes positively correlate with lysis rates (Ameh et al., 2020), the propagation of these phages could be favoured during the isolation process that involved an enrichment step. However, G4 phages that also form big plaques were isolated from fewer environmental samples. Therefore, phages of cluster G2 are probably more widespread in the different sampling environments than G4 ones. The repeated isolation of genus cluster G5 phages with average-size plaques is more surprising. As our isolation experiments show the greatest abundance of these phages, they seem to be the most frequent culturable *P. putida* phages in environmental samples. Still, to rule out that the enrichment phase of the isolation method was the cause of the recurrent appearance of certain phages, we also carried out phage isolation without the enrichment step by directly plating phages precipitated from environmental samples. The G5 phages were still the most frequently detected in the samples, as was determined by G5-specific PCR analysis (data not shown). From that, we conclude that the G5 is indeed a prevalent cluster of *P. putida* phages in the environment. Interestingly, the G5 phages represent an entirely new genus cluster (Figure 2A). We hypothesize that the reasons for them not having been isolated previously are their very narrow host range (Figure 5A,D) and a temperature-sensitive infection efficiency (Figure 3B, Figure S4, Supplementary File 3), which means that they would have been missed when using different *Pseudomonas* hosts or higher than 25°C growth temperature for the isolation.

All phages in the current CEPEST collection are tailed double-stranded DNA phages from the order *Caudovirales*. Over three-quarters of all the 67 phages have podovirus morphology, and two genus clusters (G1 and G3) are myoviruses. Surprisingly, we did not isolate any siphoviruses, although the morphology is common for *Escherichia coli* phages (Korf et al., 2019; Maffei et al., 2021; Nicolas et al., 2023; Olsen et al., 2020). Moreover, the representation of morphotypes of *P. putida* phages in the current collection remarkably differs from that in several coliphage collections: around 70% of lytic coliphages are myoviruses, 22–25% siphoviruses and less than 10% podoviruses (Korf et al., 2019; Olsen et al., 2020). Interestingly, if we surveyed the distribution of virion morphotypes of previously isolated *P. putida* phages, only myo- and podoviruses were found in accordance with our results. Out of 13 sequenced phages that have been isolated with different *P. putida* strains, 4 had no morphology data, but 6 were podoviruses (Glukhov et al., 2012; Lee & Boezi, 1966; Ngiam et al., 2022), and 3 myoviruses (Jaryenneh et al., 2023; Magill et al., 2017). Thus, it seems that *P. putida* phages inherently differ from the phages infecting *E. coli* with podovirus morphotype being much more prevalent.



The CEPEST collection phages are highly dependent on infection temperature

Most CEPEST collection phages seem to be adapted to LTs, as their optimum infection temperature was 15–20°C, and many failed to lyse *P. putida* at higher temperatures (Figure 3). This is not surprising, given that *P. putida* is an environmental bacterium that usually meets its natural phage enemies in soil and water at much lower temperatures than 30°C that is routinely used in the laboratory for growing *P. putida*. Previous studies have shown that temperature may drastically influence the efficiency of phage infection (Seeley & Primrose, 1980). For instance, coliphages have been divided into three groups according to their temperature sensitivity: high-temperature (HT) phages that lose infectivity at or below 25°C, LT phages that lose infectivity at or above 30°C and mid-temperature (MT) phages that are infectious in the range 15–42°C (Seeley & Primrose, 1980). It has been shown that the temperature profile of phages depends on their origin: phages originating from warm-blooded animals tend to be MT or HT type, while LT-type phages can be found in aquatic habitats (Lee & Boezi, 1966). As our environmental samples originate from the temperate climate of Estonia, it is not surprising that many CEPEST phages displayed an even higher infection efficiency at 15°C compared to the isolation temperature of 20°C (Figure 3).

Many phages showed highly pronounced changes in the infection efficiency upon slight temperature changes (e.g., the 9B phages infecting very efficiently at 20°C, but unable to infect at 22°C, Figure 3). The strong effects of phage infection often result from the phage's inability to enter the bacterial cells. As the surface layers and features of *P. putida* are suggested to alter drastically upon prolonged growth at LTs, for example, the expression of different porins, transporters, and proteins increases (Fonseca et al., 2011), it is possible that these changes may accordingly allow (at LTs) or impede (at higher temperatures) phage adsorption or genome injection. The evident LT requirement of many *P. putida* phages is very encouraging when considering *P. putida* as a promising microbial cell factory for biocatalysis. It seems that besides its versatile metabolism and high-stress tolerance that make *P. putida* a valuable candidate for bioproduction, its increased phage resistance at higher temperatures when metabolic reactions are more efficient adds value to its use in bio-industrial applications.

The host and receptor specificity of the CEPEST collection phages

The majority of the CEPEST collection phages display a narrow host range and could not infect other

Pseudomonas species or other *P. putida* strains, except for the *P. putida* PaW85 ancestor strain mt-2 (Figure 5). Still, some phages, particularly those from *Pifdecavirus* (G2) and *Ghunavirus* (G4, G7, G9) genus clusters, had a broader host range, as they could lyse several other *P. putida* and even *P. syringae* strains. Given that the host specificity is very often determined by the level of receptor recognition, it is interesting to note that the only phages able to infect the rough cells of the $\Delta wbpL$ strain were from the genus clusters with a broader host range. Phages from genus cluster G2 had a wide host range and could also infect the O-antigen deficient strain $\Delta wbpL$ (Figures 5 and 6). However, opposite to G2 cluster, the phages from genus clusters G4 and G7 that could infect the O-antigen deficient strain, that is, Emajogi (4A) and Kompost-2 (7B), had a narrow host range, whereas the other species in these clusters, Luke-2 (4B) and Kallioja (7A) with a wider host range, were unable to infect the $\Delta wbpL$ strain (Figures 5 and 6). The infection pattern of *Ghunavirus* Kompost-2 is unique in the collection, as the infection is more efficient when the O-antigen is missing. This suggests that the O-antigen probably masks the main receptor of the phage on the wild-type cells, and the receptor is more accessible upon O-antigen deletion. This all suggests that the receptors of the phages able to infect the $\Delta wbpL$ cells are different. For tailed phages, receptor recognition is usually determined by the C-terminal parts of tail fibre proteins, and it is quite common for these areas of the proteins to undergo shuffling as one of the processes that diversify the host range of the phages (Smug et al., 2023). This can lead to otherwise similar phages having diverse receptors or varied host ranges. The *Ghunavirus* cluster G4 of the CEPEST collection illustrates this well, as both the host range and the receptor specificity differ between species 4A and 4B (Figures 5 and 6), although their full genome sequences are almost 93% identical (Table S7, Supplementary File 2).

P. putida genomic prophages and plasmid pWW0 confer defence against certain CEPEST collection phages

The CEPEST collection contains only phages that can overcome the putative antiphage systems of *P. putida*. The only exceptions are the potential defence provided by the TA systems and cryptic prophages that were deleted from the genome of the isolation host strain (Figure 1). Our previous efforts have not detected any importance of *P. putida* chromosomal TA systems for bacterial physiology (Rosendahl et al., 2020). However, due to the lack of *P. putida* phages, we could not previously test the possible effect of TA systems in phage defence. Here, we compared the phage resistance of wild-type *P. putida* PaW85 and its 13 TA system



deletion derivative but did not detect any differences in the phage defence of the two strains (Figure 4A,B). Our previous data have revealed that several TA systems encode for nontoxic proteins (Rosendahl et al., 2020), which indicates that these toxins have probably lost their function even if they once would have participated in phage defence. Nevertheless, 8 out of the 13 toxins deleted from *P. putida* were moderately toxic or lethal proteins (Rosendahl et al., 2020) and still did not affect the phage resistance of the bacterium. This means that none of the phages in the current CEPEST collection can activate any of the 13 TA systems in *P. putida*. However, the 22 species and 67 isolates of phages represent a very small sample size of all possible phages. Thus, from these results, we cannot rule out the possibility that some *P. putida* TA systems could be active against yet unknown phages. As the collection is upgraded, the TA systems' involvement in phage infection will be kept under consideration. Yet, current results demonstrate that the 13 chromosomal TA systems provide no general phage defence and neither act as specific phage defence systems against the 67 tested bacteriophage isolates of the CEPEST collection.

Differently from TA systems, however, the four cryptic prophages can protect *P. putida* at variable strength against about half of the 22 species of phages. Our future work will determine whether this protection is provided by a single prophage or several prophages together. It is known that prophages may inhibit the expression of lytic genes from related phages (Susskind et al., 1974). As prophages keep their genes for lytic development repressed, the infection of phages that share similar repressors is also inhibited (Bondy-Denomy et al., 2016). Yet, as our analysis of the prophage regulator or any other prophage genes revealed no homology among any of the phages in the CEPEST collection (data not shown), this type of defence does not seem to be the mechanism of protection by these prophages. Aside from suppressing the lytic development of related phages, prophages can carry various defence genes that protect the host from attacks by other phages. For example, the superinfection exclusion systems either inhibit the adsorption of new phages (Bondy-Denomy et al., 2016) or prevent their genome injection (Cumby et al., 2012; Hofer et al., 1995; Susskind et al., 1974). Prophages can also encode TA gene(s) or other Abi systems (Owen et al., 2021; Zhang et al., 2022) that prevent the spread of the exogenous phages in bacterial populations by inducing the death of the first infected cell. Our next efforts will be directed towards identifying these systems to pinpoint the mechanisms of the prophage-provided defence.

The studies of host specificity of the CEPEST collection phages led to the discovery of the protective effect of the TOL plasmid pWW0 (Assinder &

Williams, 1990; Greated et al., 2002; Ramos et al., 1997; Worsey & Williams, 1975) against the phages from genus cluster G5 (Figure 5C). It has been shown that plasmids may influence the efficiency of phage infection by either plasmid-encoded factors that are required for phage entry (Haase et al., 1995; Olsen et al., 1974) or, on the contrary, strongly decreasing the infection efficiency by plasmid-coded defence systems (Emond et al., 1998). The pWW0 plasmid-encoded pili have been shown to support the infection of plasmid-dependent phages PR4 and PRD1 (Bradley & Williams, 1982), but to our knowledge, the pWW0 plasmid has never been associated with phage defence. According to the NCBI database (Sayers et al., 2022), two genes on the TOL plasmid (NC_003350) are annotated as TA genes: HXE35_RS00070 as type II TA system RelE/ParE family toxin and HXE35_RS00065 as addiction module antidote protein (annotation as on 3 May 2024). This system will be tested for involvement in the TOL-provided phage defence. As another option, the presence of the TOL plasmid in the cell could somehow change the phage resistance indirectly. The exact mechanisms for the protection provided by the TOL plasmid are subjects of further research using the diverse and well-represented genus cluster G5 (Table S3, Supplementary File 2) of the CEPEST collection.

CONCLUSION

Here, we present the first broad collection of phages infecting the environmental bacterium and model organism *P. putida* PaW85. The collection is built up of phages isolated against a host whose phage resistance is completely unknown. The results obtained on the phage defence during the screening stages will be followed up with research behind the mechanisms of the observed biological effects. Thus, the CEPEST collection allows the phage resistance research of this widely used model bacterium. As knowledge of the phage defence of *P. putida* broadens, more phages will be isolated using weakened *P. putida* strains as hosts to increase the diversity of the CEPEST collection and improve the understanding of the interactions between *P. putida* PaW85 and its phages.

AUTHOR CONTRIBUTIONS

Age Brauer: Writing – original draft; writing – review and editing; data curation; formal analysis; visualization; methodology; investigation; validation; software. **Sirli Rosendahl:** Investigation; writing – original draft; methodology; validation; visualization; writing – review and editing; formal analysis; supervision; data curation. **Anu Kängsep:** Investigation; validation. **Alicja Cecylia Lewańczyk:** Investigation; validation. **Roger Rikberg:** Investigation; validation. **Rita Hörak:** Conceptualization; funding



acquisition; writing – original draft; investigation; methodology; validation; writing – review and editing; formal analysis; project administration; supervision; resources. **Hedvig Tamman:** Conceptualization; investigation; funding acquisition; writing – original draft; methodology; validation; visualization; writing – review and editing; formal analysis; project administration; supervision; resources.

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CONFLICT OF INTEREST STATEMENT

The author declares that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

Genomes are openly available in GenBank under BioProject PRJNA1067406: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1067406>.

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