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Research Article

Coevolutionary phage training and Joint application delays the emergence of phage resistance in *Pseudomonas aeruginosa*

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Abstract

Antibiotic-resistant bacteria are current threats to available antibiotic therapies, and this has renewed interest in the therapeutic use of phage as an alternative. However, development of phage resistance has led to unsuccessful therapeutic outcomes. In the current study, we applied phage training to minimize bacterial phage resistance and to improve treatment outcome by adapting the phage to their target hosts during co-evolution. We isolated and characterized a novel *Pseudomonas aeruginosa* N4-like lytic phage (PWJ) from wastewater in Yangzhou, China. PWJ is a double-stranded DNA podovirus that can efficiently lyse the model strain ATCC 27,853 and opportunistic pathogen PAO1. Genome sequencing of PWJ revealed features similar to those of the N4-like *P. aeruginosa* phage YH6. We used PWJ to screen for an evolved trained phage (WJ_Ev14) that restored infectivity to PWJ phage bacterial resisters. BLASTN analysis revealed that WJ_Ev14 is identical to its ancestor PWJ except for the amino acid substitution R1051S in its tail fiber protein. Moreover, phage adsorption tests and transmission electron microscopy of resistant bacteria demonstrated that the R1051S substitution was most likely the reason WJ_Ev14 could re-adsorb and regain infectivity. Furthermore, phage therapy assays *in vitro* and in a mouse *P. aeruginosa* lung infection model demonstrated that PWJ treatment resulted in improved clinical results and a reduction in lung bacterial load whereas the joint phage cocktail (PWJ+ WJ_Ev14) was better able to delay the emergence of resister bacteria. The phage cocktail (PWJ +WJ_Ev14) represents a promising candidate for inclusion in phage cocktails developed for clinical applications.

Keywords: *Pseudomonas aeruginosa*; coevolution; antimicrobial-resistant (AMR); phage therapy; phage training; phage resistance.

1. Introduction

Clinical application of antimicrobials has saved millions of lives since their discovery in 1920s. Unfortunately, antimicrobial-resistant (AMR) bacteria now threaten antimicrobial effectiveness (Santajit and Indrawattana 2016) and the current clinical development pipeline for antimicrobials is lagging far behind AMR development (Tyers and Wright 2019; Theuretzbacher et al. 2020). Bacteriophage therapy (BT) can be an antimicrobial alternative or supplement to address the increase in AMR bacteria (Matsuzaki et al. 2014; Wang et al. 2023a). Phages were initially described in 1915 and 1917 (Twort 1915; D'Herelle 1916) and the bacteriologist Felix d'Herelle first coined the term 'bacteriophage' and began using phage to treat bacterial infections (Duckworth 1976).

Bacteriophages (phages) are obligate bacterial parasites with specific host ranges (Hatfull and Hendrix 2011) and represent the most abundant biological entities on Earth (Suttle 2007; Paez-Espino et al. 2016; Dion, Oechslin, and Moineau 2020). Phages are also abundant within humans and animals (Cao et al. 2022; Tiamani et al. 2022) and play key ecological roles in regulating the microbial community within these niches (Liang and Bushman 2021). Phages can therefore provide a source of raw materials for clinical applications. The current methods of phage-driven and inspired therapeutic strategies have shown promising results (Marongiu et al. 2022) and have been utilized to treat clinically important priority pathogens such as *Pseudomonas aeruginosa* (Aslam et al. 2019; Ferry et al. 2022) and *Acinetobacter baumannii* (Wu et al. 2021; Chen et al. 2022). However, these types of clinical trials have been influenced by the development of phage resistance imposed by the host bacteria (Labrie, Samson, and Moineau 2010).

One prerequisite for successful BT is that the phage must complete the lytic life cycle of surface receptor adsorption, genome injection, virion assembly, and virion release (Wang et al. 2023b). Each of these steps is vulnerable to host-evolved resistance (Egido et al. 2022) and there are three primary categories for host resistance development (Egido et al. 2022). The first one is receptor adaptations including receptor point mutations, receptor masking such as outer-membrane vesicle production, and extracellular matrix production and phase variation. The second is host defense systems known as bacterial immunity (Tal and Sorek 2022) including restriction-modification (R-M) systems, CRISPR-Cas immunity, defense island system-associated R-M, an epigenetic modification bacterial exclusion system, and the Argonaute microRNA pathway as well as abortive infections and secondary metabolite production. Phage-derived defense systems can also lead to infection failure that includes superinfection exclusion (Chernov et al. 2019). Although the failure of phage therapy was not always related to the phage resistance emergence (Schooley Robert et al. 2017; Blasco et al. 2023; Jean-Paul et al. 2023), phage resistance has been recognized as a concern during phage therapy, and the avoidance of phage-resistant bacteria emergence also has not been extensively investigated. Hence, novel phage-derived therapeutic strategies are needed to mitigate the evolution of phage resistance (Oromi-Bosch, Antani, and Turner 2023).

A recent report has outlined the use of evolved or pre-adapted/trained phage to circumvent resistance development (Rohde et al. 2018). Evolved phages have been shown to be more effective than their ancestral parents in chronic cystic fibrosis *P. aeruginosa* chronic isolates of the lung and better reduced the densities of chronic bacterial isolates (Friman et al. 2016). Trained λ phages were also able to suppress bacterial growth 1,000-fold greater and for 3 to 8 times longer than the untrained ancestor *in vitro* (Borin et al. 2021). Also, the *Pseudomonas* phage K8 evolved a broader host range by acquiring a T239A mutation in its baseplate wedge protein gene GP075 and this led to a higher killing efficiency (Sun et al. 2023). Moreover, previous literatures have shown that increased phage genetic diversity inhibits bacterial evolution (Broniewski et al. 2020; Castledine et al. 2022), implying an increased therapeutic efficacy. On the other hand, if evolved phage is selected by using ancestral phages to infect evolved resistant strains which originated from the host strain of ancestral phages, the joint application of evolved phage and ancestral phage as a cocktail to treat the host strain will be further enhanced because of the combination cocktail can stop the host bacteria to the most adaptive state of evolution, forcing an increase in the fitness cost and improvement in the treatment efficacy.

In this study, we isolated and characterized a novel *P. aeruginosa* N4-like lytic phage (PWJ) from wastewater in Yangzhou, China that could efficiently lyse the opportunistic pathogen *P. aeruginosa* PAO1. We also evolved this phage *in vitro* and the resultant progeny (WJ_Ev14) restored infectivity to PWJ phage resisters. An R1051S mutation in its tail fiber protein was also correlated with its greater infectivity. Moreover, a mouse model of *P. aeruginosa* lung infection demonstrated that the phage cocktail (PWJ/WJ_Ev14) was able to additionally delay the emergence of resister bacteria. Therefore, trained phage included with the ancestral phage as a cocktail will be a promising choice and should be included in the BT toolbox.

2. Materials and methods

2.1 Strain and phage collection

P. aeruginosa strain ATCC 27,853 (NZ_CP011857) and *P. aeruginosa* opportunistic pathogen PAO1 (AE004091) were used for this study. A novel PWJ was isolated from wastewater in Yangzhou, China and was passed through strains ATCC 27,853 and PAO1 using methods previously described for *Escherichia coli* strain C600 (Van Twest and Kropinski 2009; van Charante et al. 2019; Luong et al. 2020; Wang et al. 2023b). The taxonomy ranks were as follows: Realm, *Duplodnaviria;* Kingdom *Heunggongvirae;* Phylum *Uroviricota*; Order *Caudoviricetes;* Family *Schitoviridae;* Subfamily *Migulavirinae;* Genus *Litunavirus.* Briefly, the wastewater samples were filtered through a Millex-GP 0.22 μm membrane (Millipore, Burlington, MA, USA) and the filtrate was incubated with *P. aeruginosa* strains ATCC 27,853 and PAO1 overnight at 37°C with shaking at 200 rpm. The newly discovered phage PWJ detected from spot testing and single plaques were selected, purified, and diluted in SM buffer (100 mM NaCl, 8 mM MgSO4, 50 mM Tris-HCl pH 7.5) using the double-layer soft agar plate method and stored at 4°C.

2.2 Culture-based phage resister selection and coevolved phage screening

2.2.1 *Screening of phage-resistant mutants*

Bacterial adaptation to phage PWJ was experimentally performed with three replicates as previously described (Gurney et al. 2020; Oyejobi et al. 2022) with a slight revision. Briefly, in 2 mL LB medium, 200 μL fresh lysate of phage PWJ and 200 μL ATCC 27,853 from a single ancestral *P. aeruginosa* colony were incubated together at 37°C at a multiplicity of infection (MOI) of 1. In 48 h intervals, samples were taken and centrifuged at 2700 × g and the cell pellets were suspended in fresh LB that was then innoculated with phage PWJ at MOI = 1 and incubated again for another interval. This process was repeated for five transfers over 10 d. At each transfer, bacterial CFU and phage PFU were measured and a total of 30 evolved mutants were also randomly selected. Following the final interval, the bacterial were again pelleted and suspended in LB and spread-plated on LB agar to recover bacterial survivors. Spot assays and cross streaking against the ancestral phage were then used to determine phage PWJ resistance in the evolved bacteria.

2.2.2 *Phage training*

Experimental phage counteradaptation to phage-resistance was experimentally performed with three replicates as previously described (Akusobi et al. 2018) with a slight revision. Briefly, in 1 mL LB medium, a 20 μL of stationary-phase phage-resistant mutant culture was combined with 20 μL ATCC 27,853 from a single ancestral *P. aeruginosa* colony incubated together at 37°C until the OD600nm reached ∼0.3. A 200 μL sample of a fresh PWJ lysate was added followed by incubation at 37°C for 5 h. The mixed samples were filtered through a 0.22 μm membrane twice and one aliquot was used for another round of adaptation. The other aliquot was used for spot assays and cross streaking against the ancestral bacteria and phage resistant mutant.

2.3 Biological characteristics of *Pseudomonas* phage PWJ and WJ_Ev14

2.3.1 *Transmission electron microscopy*

Phage PWJ or WJ_Ev14 was concentrated 50-fold using a 100 kDa Amicon Ultra centrifugal filter units (Millipore) and 15 μL of phage concentrate was dropped onto carbon-coated formvar covered grids and allowed to stand for 15 min. The preparations were then stained with 2 per cent w/v phosphotungstic acid pH 7.0 and air-dried. PWJ was then examined using a FEI transmission electron microscope (Thermo Fisher, Hillsboro, OR USA) at an acceleration voltage of 80 kV.

2.3.2 Optimal MOI

The optimal MOI was determined in an appropriate susceptible host cultured to early log phase growth and incubated with phage in the following ratios: PWJ (or WJ_Ev14): ATCC 27,853 = 100:1, 10:1, 1:1, 0.1:1 and 0.01:1). Mixtures were cultured at 37°C for 5 h. Phage titers were calculated for each group using the double-layer soft agar plate method in triplicate. Phage PFU per mL were determined by using spot assays as previously described (Zhang et al. 2021).

2.3.3 Phage growth curve

The Phage PWJ or WJ_Ev14 was mixed with exponential phase ATCC27,853 (1×10^8 CFU/mL) at the MOI of 1 and allowed to absorb to ATCC 27,853 cells for 10 min at room temperature. The mixture was centrifuged at 2,700 g for 10 min to remove unabsorbed phages and infected cells were resuspended with LB to 5 mL. The culture samples were harvested every 10 min. The culture samples were centrifuged and filtered by 0.22um filter membrane, and the phage titers in the samples were calculated using the double-layer agar method. This experiment was performed in triplicate.

2.3.4 Thermostability and pH Tolerance

Phage PWJ and WJ_Ev14 thermostability was examined by incubating phage stocks ($\geq 10^8$ PFU/mL) at 4, 37, 50, 55, 60, and 70°C for 1 h and pH tolerance was examined by incubating over a pH range of 1.0 to 12.0 for 1 h at 37°C. Phages were determined by double-layer agar plate as above with three duplications.

2.3.5 Phage adsorption assay

Quantification of Phage PWJ and WJ_Ev14 adsorption to bacterial cells was performed by mixing overnight bacterial cultures and phage from fresh lysates at MOI = 1. Suspensions were collected at 10, 20, and 30 min and centrifuged at 2,700 g for 3 min. Free phage particles were determined by double-layer agar plate as above with three duplications.

2.4 Genomic characterization of *Pseudomonas* phage PWJ and WJ_Ev14

2.4.1 DNA Extraction

Purified phage lysates were concentrated by incubation with PEG 8000 and then treated with DNase I (Sangon Biotech, Shanghai, China) at 100 U/mL prior to DNA extraction to eliminate potential DNA contamination. Phage DNA extraction was conducted according to the previous study (Thurber et al. 2009; Wang et al. 2018, 2021; Luong et al. 2020). An aliquot of a phage lysates was amplified for bacterial 16S rDNA determinations using PCR to eliminate bacterial DNA contamination and only negative samples were used for the subsequent sequencing. The extracted DNA was then suspended in TE buffer and DNA concentrations were determined by UV spectroscopy (NanoDrop, Wilmington, DE, USA).

2.4.2 Genome sequencing and assembly

The phage genomic DNA of phage PWJ and the evolved WJ_Ev14 was subjected to short-read sequencing (2 × 150 bp) with the Illumina HiSeq 2500 platform at Genewiz Biological Technology (Suzhou, China). DNA was also sequenced with the Oxford Nanopore Technologies MinION long-read platform with the RBK004 barcoding library preparation kit and MinION R9.4.1 flow cells to obtain the complete sequences as described previously (Li et al. 2020). Short-read Illumina raw sequences were separately assembled into contigs using SPAdes (Bankevich et al. 2012) and contigs < 500 bp were discarded. The Flye long-read assembly tool was used to perform *de novo* assembly of Nanopore long-read MinION sequences of phage genomic DNA.

2.4.3 Genome annotation

The complete genome sequences of PWJ and WJ_Ev14 were annotated using RAST (http://rast.nmpdr.org/) automatically and modified manually. Briefly, after comparisons with protein sequences of known function using BLASTP with an E-value of $\leq 1 \times 10^{-5}$ and an identity of ≥ 70 per cent (Brettin et al. 2015), the best hits were selected for the annotation of the gene products. CGview Server (http://cgview.ca/) was used to draw the whole genome map of the PWJ and evolved WJ_Ev14.

2.4.4 Genetic polymorphism analyses

The evolutionary relationships between PWJ and other N4-like phages were analyzed using phylogenetic trees based on the whole genome against the NCBI representative RefSeq database (Menon et al. 2021), amino acid sequences of the terminase large subunits (Black 2015), and the N4-like phage specific virion-associated RNA polymerases (RNAP) (Ceyssens et al. 2010). Phylogenetic analysis was performed with MEGA7 by the neighbor-joining method using 1000 bootstrap replications with the Poisson model (Yang et al. 2020a).

2.5 Biofilm experiment

Biofilm experiment was conducted as previously described (Peeters, Nelis, and Coenye 2008). Briefly, a 100 μL aliquot of LB broth was inoculated with 10 μL of bacterial solution (strains 27,853, M11, and M12) in a 96-well plate. The initial bacterial concentration was adjusted to 10∧6 CFU/mL. The plate was then placed in a static incubator at 37°C for 24 hours for bacterial growth. Then, the liquid culture in the 96-well plate was carefully aspirated and discarded. Subsequently, 50 μL of methanol was added to each well for fixation, and the plate was left undisturbed for 15 minutes. After fixation, the methanol was aspirated, and the plate was allowed to air-dry. For staining, each well was filled with 100 μL of a 0.1 per cent crystal violet solution for 15 minutes. After staining, the crystal violet solution was aspirated, and the plate was thoroughly washed with PBS several times before being allowed to air-dry. To dissolve the crystal violet dye, 100 μL of a 33 per cent acetic acid solution was added to each well, and the plate was incubated at 37°C for 30 minutes. The resulting solution was used to measure absorbance at 570 nm to assess bacterial biomass and biofilm formation.

2.6 Bacterial suppression experiment of PWJ and WJ_Ev14 in vitro

The trained WJ_Ev14 and untrained PWJ phage were compared for their abilities to suppress ATCC 27,853 and PAO1 growth and was conducted as previously described with a minor revision (Borin et al. 2021). Strains ATCC 27,853 and PAO1 were used to determine whether and when resistance evolved in each of three treatment groups. Briefly, six 50 mL flasks were inoculated with 10^6 ATCC 27,853 cells in 10 mL M9. Three flasks were inoculated with PWJ ($\sim 10^6$ particles) and the other three with WJ_Ev14 ($\sim 10^6$ particles) and incubated at 37°C with shaking at 120 rpm. Every 24 h, 100 μL of each flask was transferred into new with 10 mL of fresh media. The experiment was repeated for 15 consecutive days. Each day, bacterial and phage densities were estimated aliquots were preserved for later analyses. The bacterial and phage titers were

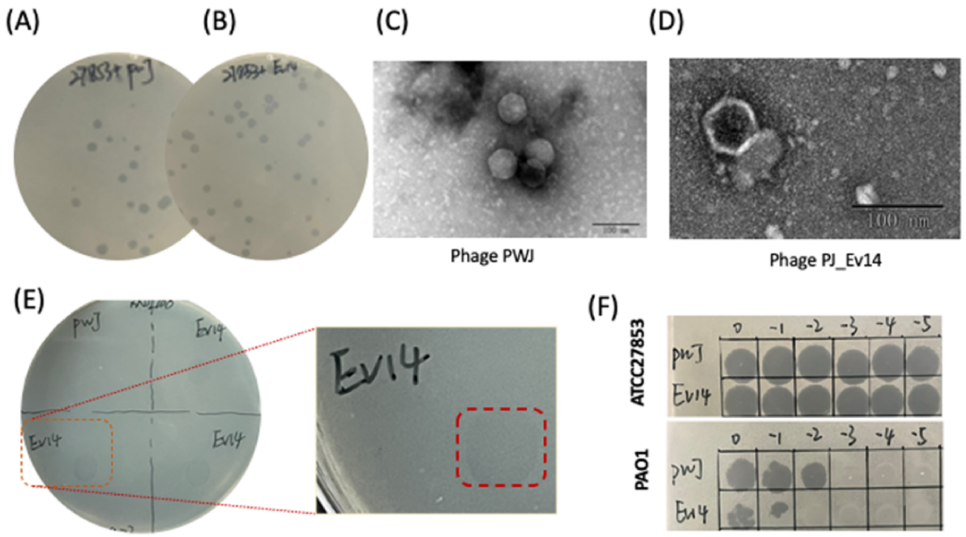

Figure 1. Morphological characteristics of lytic phage PWJ and PJ_Ev14. Culture maps of (A) phage PWJ and (B) phage PJ_Ev14 against host strain ATCC27,853, plated by a double-layer agar method to visualize individual plaques. TEM images of (C) PWJ and (D) PJ_Ev14. (E) Spot assay map of phage PJ_Ev14 for the PWJ resister bacterial strain PWJ^{M1}, showing that restored infectivity to PWJ phage bacterial resisters. (F) Efficiency of plaque assay of phage PWJ and phage PJ_Ev14 for *P. aeruginosa* strains ATCC 27,853 and PAO1.

also calculated, and the proportions of sensitive, partially resistant, and completely resistant bacterial populations were also calculated.

Bacterial resistance to phage lysis was examined for 20 isolates every 2 days to quantify the time required for the emergence of resisters (delay time). A resistance ratio was determined by counting the resisters using phage spot assays for cultures growing with PWJ alone, with PWJ plus WJ_Ev14 group, and without phage. Bacteria were deemed sensitive if no growth was observed in the presence of phage. Partial resistance was recorded for bacteria that showed signs of growth but were clearly inhibited by the phage and completely resistant if cells grew uninhibited by phage.

2.7 Bacterial suppression experiment of PWJ and WJ_Ev14 in mouse lung model

A mouse lung model of bacterial infection was performed as per our previous study (Liu et al. 2020). Briefly, the Center of Comparative Medicine in Yangzhou University provided 6-8 week-old BALB/c mice. The animal study protocols were performed in accordance with the guidelines and regulations (ID: SCYK2017-0007). The experiment was performed to determine whether joint application of ancestral phage PWJ and trained phage WJ_Ev14 would better suppress and delay *P. aeruginosa* growth than a single dose of PWJ. PAO1 was used to intranasally infect 4 BALB/C mice per group with 1.0×10^8 CFU PAO1 and 2 h post-infection (hpi), mice were treated with sterile water (Group A), phage PWJ (Group B, MOI = 1) alone or a combination of PWJ plus WJ_Ev14 (Group C, MOI = 1) via intranasal injection. Bacterial titers in treated mice were determined from lung at 8, 16, 24, 48, and 96 h, and lung tissues were collected for histological hematoxylin and eosin staining.

2.8 Statistics

Statistical analysis was performed using Prism 9.0.0 (GraphPad, Boston, MA, USA). All data were presented as means ± SD. Prism's built-in one-way ANOVA and Tukey's multiple comparison test were used as appropriate to determine the effects of phage treatment and resisters (bacteria) in comparison to parental cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, ns: no significance ($P > 0.05$).

2.9 Accession number(s)

The sequences of N4-like phage PWJ (OR237807) have been submitted to the NCBI.

3. Results

3.1 Isolated phage PWJ is a *P. aeruginosa*-specific podovirus

A lytic phage was isolated from wastewater in Yangzhou, China and named *Pseudomonas* phage PWJ that was able to lyse *P. aeruginosa* model strain ATCC 27,853 as well as the opportunistic pathogen PAO1. Phage PWJ formed transparent plaques of approximately 3.0 mm in diameter on bacterial lawns (Fig. 1A). Transmission electron microscopy (TEM) observations of both the phage PWJ and WJ_Ev14 revealed an icosahedral capsid with a cross diameter of ~ 50 nm with a longitudinal diameter of ~ 50 nm (Fig. 1C). TEM observations of trained phage WJ_Ev14 revealed an overall similarity to PWJ (Fig. 1B, D). After co-culture of the phage resistance mutant PWJ^{M1} and ATCC 27,853, a co-evolved trained phage WJ_Ev14 that could re-lyse the phage-resistant strain PWJ^{M1} was isolated at day 28 (Fig. 1E and F).

3.2 Characterization of PWJ and co-evolved WJ_Ev14

The one-step growth curve of PWJ was analyzed by infecting ATCC 27,853 at MOI = 10 to ensure complete culture lysis without bacterial growth. The phage PWJ had a latent period of about 20 min and a burst period of about 50 min (Fig. 2A). MOIs ranging from 0.001 to 100 were then used to determine the optimal values for producing maximal phage titers. At MOI = 1, the phage titer was maximal (2.70×10^8 PFU/mL using a MOI of 1 (Fig. 2B). The pH and thermostability tolerance of PWJ were also determined and following 60 min incubation periods, the phage titers were stable from pH 6 to 8 but decreased by 2–3 log at pH 5, 9, or 10 and by 4 logs at pH 4 or 11. The phage PWJ had little activity in the pH ranges of 1 to 3 and 12 to 14 (Fig. 2C). Exposure of phage lysates to temperatures of –80, 4, 25, and 37°C did not alter the titers whereas the titers gradually decreased by 1 log with incubations at 50, 60, and 70°C (Fig. 2D). Interestingly, physical and chemical properties

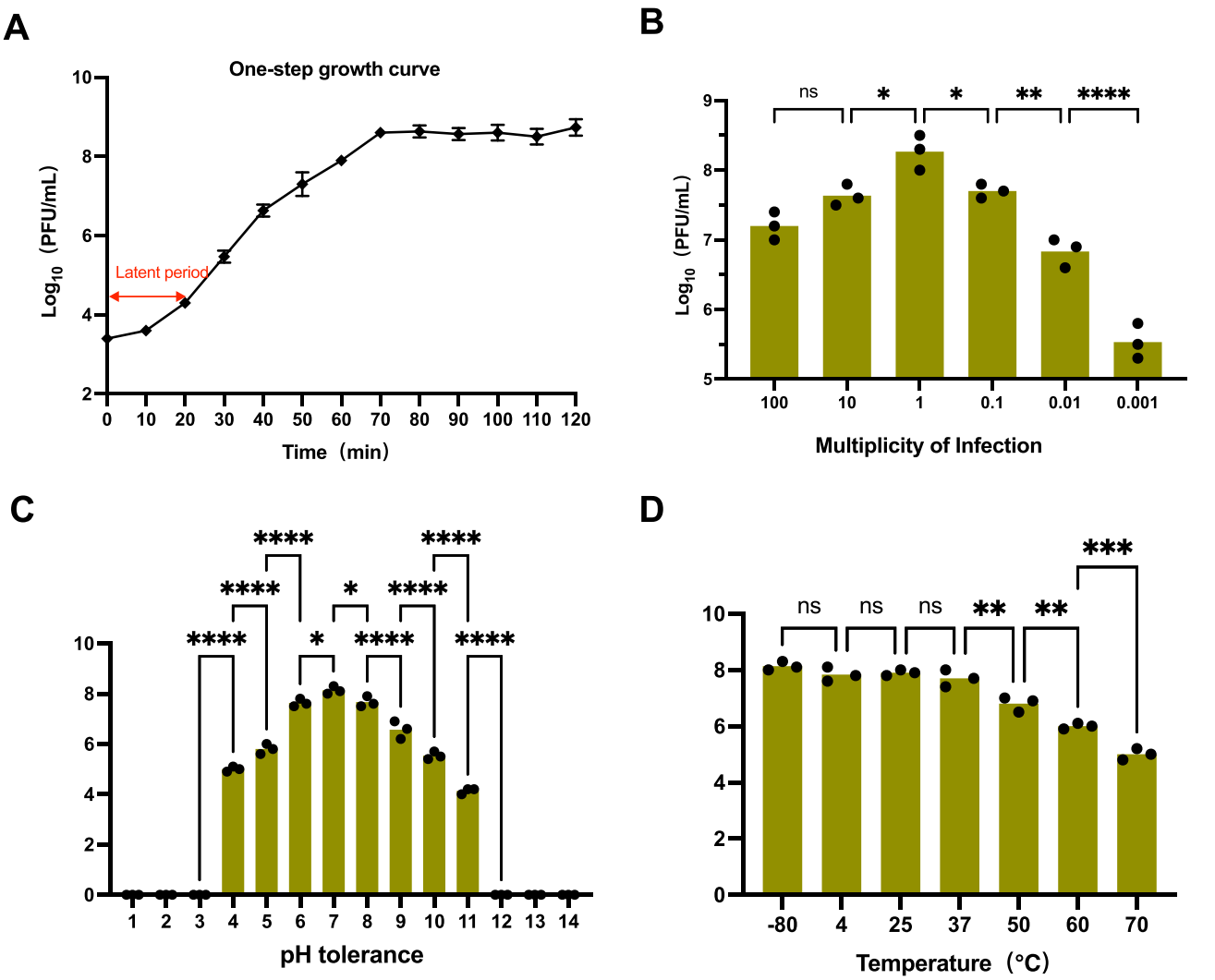

Figure 2. Physiological characteristics of PWJ. (A) PWJ one-step growth curve suggests a latent phase of 20 min for ATCC27,853 infectivity. (B) Optimal MOI determination of PWJ in the following ratios: PWJ: ATCC 27,853 = 100:1, 10:1, 1:1, 0.1:1, and 0.01:1. The MOI of 1 had the highest titer. (C) PWJ thermostability was examined by incubating phage stocks ($\geq 10^8$ PFU/mL) at 4, 37, 50, 55, 60, and 70°C for 1 h. (D) pH tolerance of PWJ was examined by incubating over a pH range of 1.0 to 12.0 for 1 h at 37°C. Phages were determined by double-layer agar plate method as above with three duplications. Significance was determined using one-way ANOVA (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, ns: no significance ($P > 0.05$)).

of PWJ and co-evolved WJ_Ev14 had a similar trend (Fig. S1 and Table S1, S2 and S3).

3.3 Bioinformatics analysis of PWJ and co-evolved WJ_Ev14

The complete genomic sequence of PWJ was submitted to GenBank under accession number OR237807. PWJ was highly similar to the N4-like *P. aeruginosa* phage YH6 (Fig. 3A). Importantly, a characteristic of N4-like genomes that of a large virion-associated RNAP vRNAP was detected (Wittmann et al. 2020). Thus, PWJ could be classified in the family *Schitoviridae*, subfamily *Migulavirinae*, and the genus *Litunavirus*. However, although the genomes of PWJ and YH6 were similar, overall, the genetic identity was not high and was even as low as 76 per cent for several gene regions. The genome of PWJ was 72,098 bp with a GC content of 54.96 per cent with 91 predicted genes and most of these genes were annotated as N4-like related proteins that again supporting the designation of PWJ as an N4-like phage (Fig. 3B).

The evolutionary relationships between PWJ and other N4-like phages were analyzed based on the whole genome (Menon et al. 2021), the terminase large subunits (Black 2015), and the N4-like phage specific vRNAP (Ceyssens et al. 2010) (Fig. 4). A BLASTN search of the PWJ genome against the NCBI database resulted in 24 phages with high identity (%) to the PWJ genome. PWJ was on an independent branch and showed a high similarity with YH6, PAP02, YH30, and LP14 (Fig. 4A). The terminase large subunit grouping also located PWJ on a significantly separated independent branch with five other phages (YH30, LP14, DL61, vB_PaeP_TUMS _P121, and vB_PaeP_4029) (Fig. 4B). Grouping by vRNAPs located PWJ with these same five phages also at a significantly independent branch. Interestingly, these six phages had a closer evolutionary relationship with N4-like *Luzseptimavirus* LUZ7 rather than the same N4-like *Litunavirus* such as YH6 (Fig. 4C).

3.4 Directed evolution guides the emergence of antiresister phage WJ_Ev14

TEM observations, phage absorption, and biofilm formation assays for resister bacterium PWJ^{M1} were conducted to identify potential evolutionary mechanisms for its evolution. TEM observations indicated that the cell surface structure of resister PWJ^{M1} changed

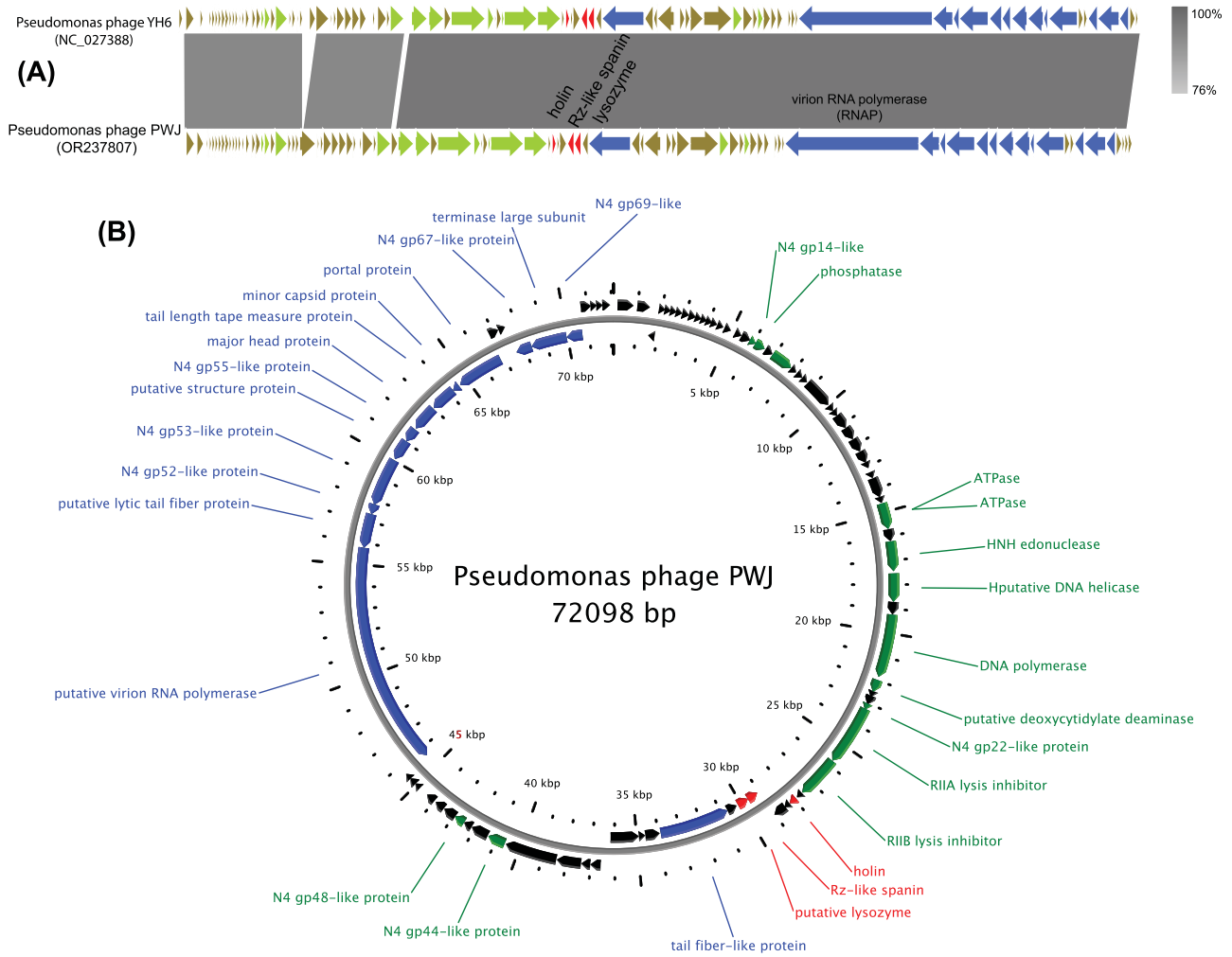

Figure 3. Bioinformatics analysis of PWJ. (A) Genome sequence alignment of phage PWJ and phage YH6. The functional modules indicated by color and similarities were shown properly according to the scale on the right corner, with replication and transcription-related genes mainly located in the left region, lytic-related gene located in the middle region, structure-related genes located mainly in the right region, and hypothetical genes scattered in different regions. (B) Annotation of the predicted genes of phage PWJ.

from smooth to rough (Fig. 5A). Furthermore, PWJ was not able to absorb to the resister PWJ^{M1} while WJ_Ev14 was still able to absorb to at a level of 80 per cent. Importantly, no significant differences were observed in the absorption rate of WJ_Ev14 when infecting ATCC 27,853 and resister PWJ^{M1} (Fig. 5B). Also, biofilm formation in strain PWJ^{M1} was significantly reduced compared with ATCC 27,853 (Fig. 5C).

To verify phage WJ_Ev14 was a PWJ derivative and to identify genetic alterations associated with its lysis ability against the resister PWJ^{R1}, we subjected the purified phage to second and third generation high-throughput whole-genome sequencing. Comparative genomic analysis of WJ_Ev14 and PWJ indicated just one SNP along the length of all 72,098 bp. The change R1051S was in the gene encoding the tail fiber protein at a location consistent with bacterial host interaction (Fig. 5D).

3.5 Bacterial suppression experiment of PWJ and WJ_Ev14 *in vitro*

The bacterial load and resistance ratio (%) were determined using ATCC 27,853 as the model strain. Application of PWJ or phage cocktails (PWJ + WJ_Ev14) could significantly reduce the bacterial load (Fig. 6A). Resistance to PWJ evolved much earlier than for the combined phage cocktails; by day 3 ≥50 per cent of isolates were partially or completely resistant to PWJ and by day 7 100 per cent of isolates were completely resistant in the PWJ group. Interestingly, despite high levels of resistance in phage cocktail, PWJ was able to persist and lyse the host bacterium until day 11 (Fig. 6B and Table S4).

When PAO1 was used as the model strain, application of PWJ or phage cocktails could also significantly reduce the bacterial load (Fig. 6C). Meanwhile, resistance in PWJ alone group also evolved much earlier than the phage cocktail group; by day 5, ≥50 per cent of isolates were partially or completely resistant to PWJ and by day 9 100 per cent of isolates were completely resistant. Also, despite high levels of resistance in the phage cocktail, PWJ was able to persist and continue lysis until day 11 (Fig. 6D and Table S5).

3.6 Bacteria burden and histological changes in the mouse lung model

P. aeruginosa strain PAO1 is a host for both PWJ and the training phage WJ_Ev14 so we used PAO1 in a mouse pneumonia model to determine if these *in vitro* results could be mirrored *in vivo*. We infected mice with PAO1 to determine whether PWJ alone

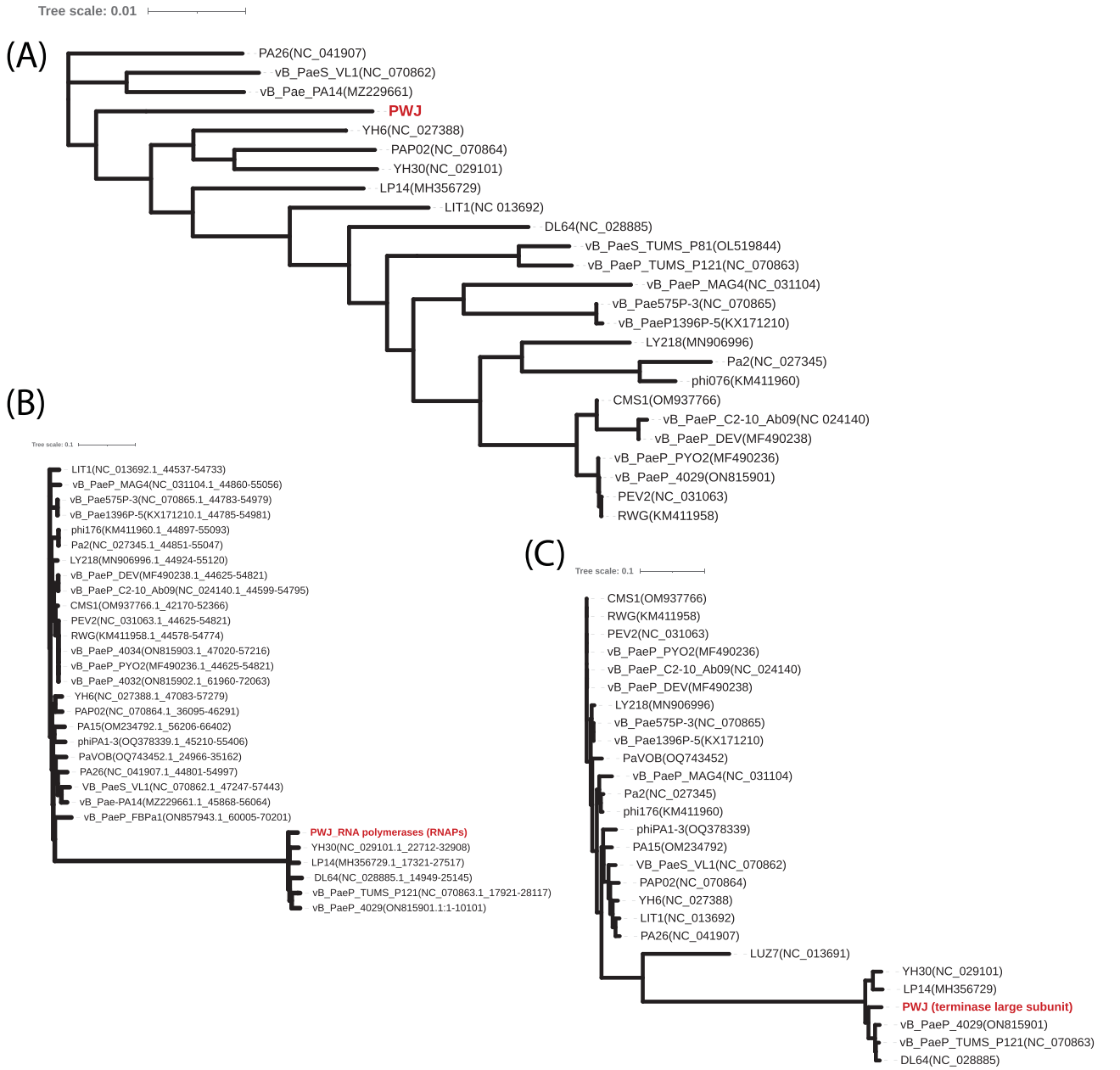

Figure 4. The evolutionary relationships between PWJ and other N4-like phages were analyzed based on the whole genome, the terminase large subunits, and the N4-like phage specific vRNAP. Analysis based on based on the (A) whole genome (B) terminase large subunit, and (C) N4-like phage specific vRNAPs.

and the cocktail could improve therapeutic efficacy and delay the development of resistant bacteria. Lung tissues from mice in the PAO1 infection control group displayed severe thickening and congestion of the alveolar walls in the perivascular and peribronchial areas compared with the untreated group after 24 h. This indicated that the mouse pneumonia model was successfully established.

Following intranasal phage treatment, lung tissues and lung homogenates were generated to calculate the bacterial burdens. PWJ treatment alone improved clinical results and reduced the lung bacterial burden. In contrast, administration of the phage cocktail was better able to significantly reduce the lung bacterial load by Day 5 (Fig. 7A). Furthermore, resistance in the PWJ alone group evolved much earlier than the phage cocktail group; by 8 hours, ≥50 per cent of isolates were partially or completely resistant to PWJ and by 24 hours 100 per cent of isolates were partially or completely resistant. Interestingly, by 8 hours, no isolates were completely resistant to PWJ and by 24 hours ≥50 per cent of isolates were still sensitive or partially sensitive (Fig. 7B and Table S6).

Histological evaluation of mouse lung tissues for the phage PWJ group demonstrated that both adventitial edema and diffuse infiltration of inflammatory cells was alleviated, and bronchial epithelial cell proliferation was also reduced compared with the PAO1 controls. However, local alveolar epithelial cells still proliferated and alveolar walls remained thickened and inflammatory infiltrates as well as inflammatory cells were present in some bronchi (Fig. S2A and Fig. S2B). Interestingly, the phage cocktail (PWJ + WJ_Ev14) was able to further alleviate both adventitial edema and diffuse infiltration of inflammatory cells and tissue

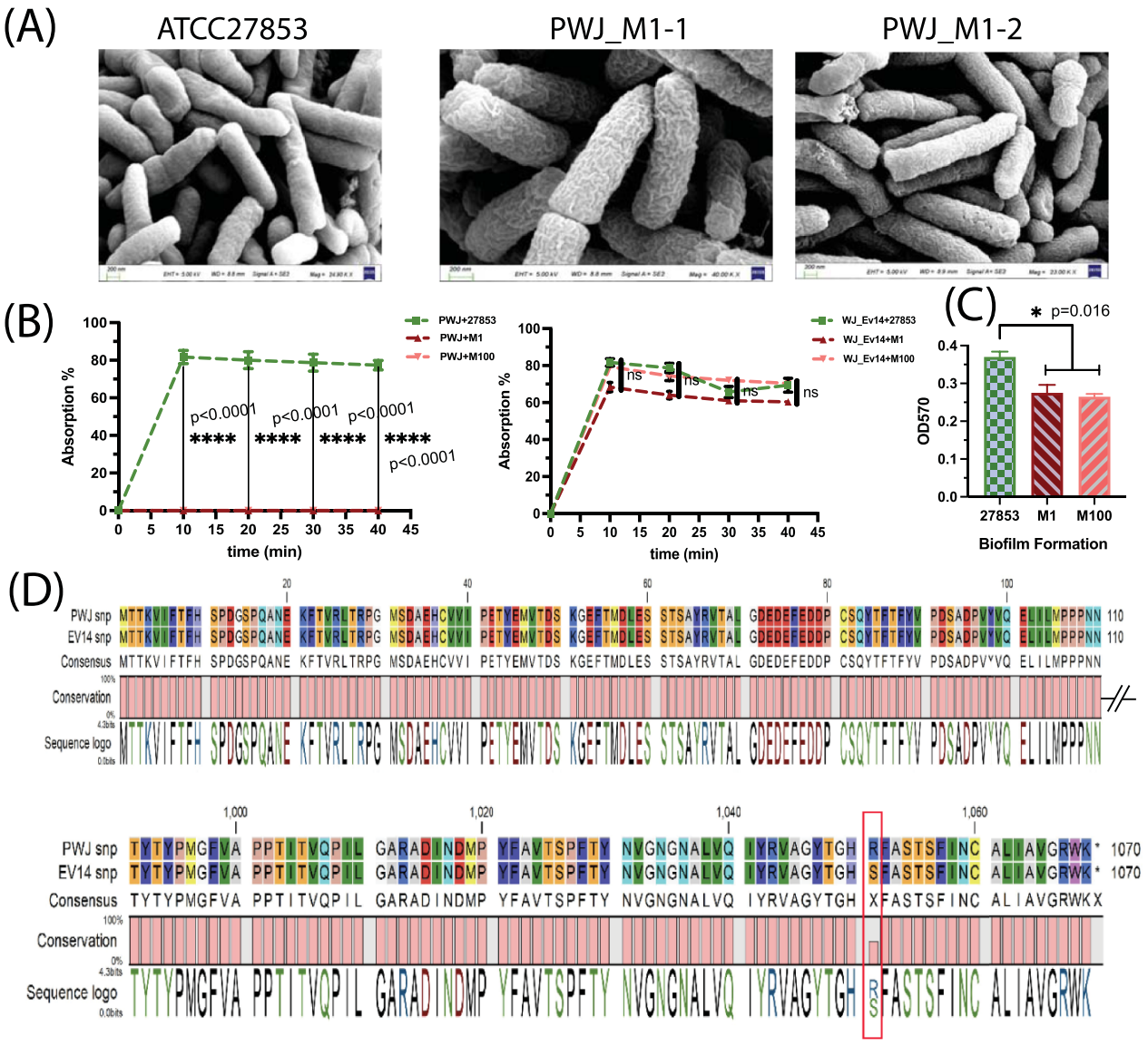

Figure 5. Characterization of PWJ and PJ_Ev14. (A) TEM observations ATCC27853 and the PWJ resister bacterial strain PWJM1. The bacteria were dropped onto carbon-coated formvar covered grids and examined using a FEI transmission electron microscope at an acceleration voltage of 80 kV. (B) Phage absorption assays. Quantification of Phage PWJ and WJ_Ev14 adsorption to bacterial cells was performed by mixing overnight bacterial cultures and phage from fresh lysates at MOI = 1 and (C) Biofilm formation. As indicated, phage PWJ, host strain *P. aeruginosa* ATCC 27853, and two PWJ resister replicates (PWJ^{M1-1} and PWJ^{M1-2}) were utilized. (D) A genetic alteration R1051S in the tail fiber protein is associated with lytic ability against resister PWJ^{R1}. Significance was determined using one-way ANOVA (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, ns: no significance ($P > 0.05$).).

Infiltration of inflammatory cells into alveolar walls as well as distribution of inflammatory cells in bronchi were also decreased (Fig. S2C).

4. Discussion

The recent rise of AMR bacterial infections has resulted in increased interest in antibiotic alternatives including the therapeutic use of phage. Regrettably, phage therapy still presents several limitations that are yet to be fully overcome. One of the most significant obstacles is the emergence of phage-resistant bacteria (Oromi-Bosch, Antani, and Turner 2023). Overcoming phage resistance can lead to improved treatment outcomes (Egido et al. 2022) and techniques of pre-adaptation of the phage to the phage-resistance bacterial during co-evolution by using phage training is one way to overcome this obstacle and guide the design of more effective phage using precision phage engineering (Borin et al. 2021).

Pseudomonas phage PWJ can lyse ATCC27,853 and PAO1 and results in transparent plaques indicating complete lysis. Also, PWJ is heat and pH stable and has a burst period < 1 h. These are attributes needed for clinical applications. Furthermore, PWJ possesses a representative vRNAP indicating it is an N4-like virus (Ceyssens et al. 2010). Phylogenetic trees of the PWJ whole genome confirmed that phages were highly conserved within the genus *Litunavirus*. Based on the sequences of the terminase large subunits (Black 2015) and vRNAP (Ceyssens et al. 2010) PWJ was related to YH30, LP14, DL61, vB_PaeP_TUMS _P121, and vB_PaeP_4029 on a significant independent branch. The phage terminase is a unique protein of dsDNA phage and possess highly conserved functions to supply energy for packaging by energizing

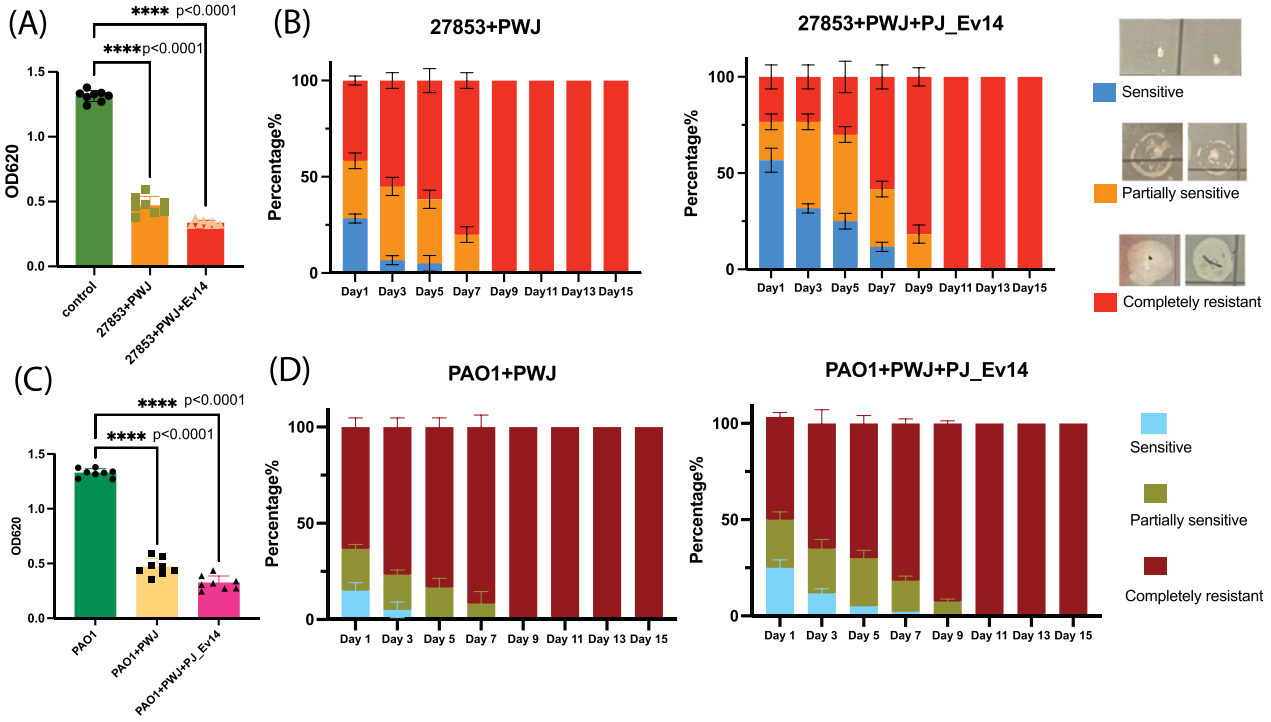

Figure 6. Resistant bacteria were counted and resistance ratios of sensitive, partial, and complete resistance were categorized by counting the resisters via phage spot assay between cultures growing with PWJ and PWJ plus WJ_Ev14 group. The experiment was repeated for 15 consecutive days and sampled at day 1, 3, 5, 7, 9, 11, 13, and 15. The bacterial load (A) and resistance ratios (B) were then determined using ATCC 27853 as the model strain. The bacterial load (C) and resistance ratios (D) were also determined using PAO1 as the model strain. Significance was determined using one-way ANOVA (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, ns: no significance ($P > 0.05$)).

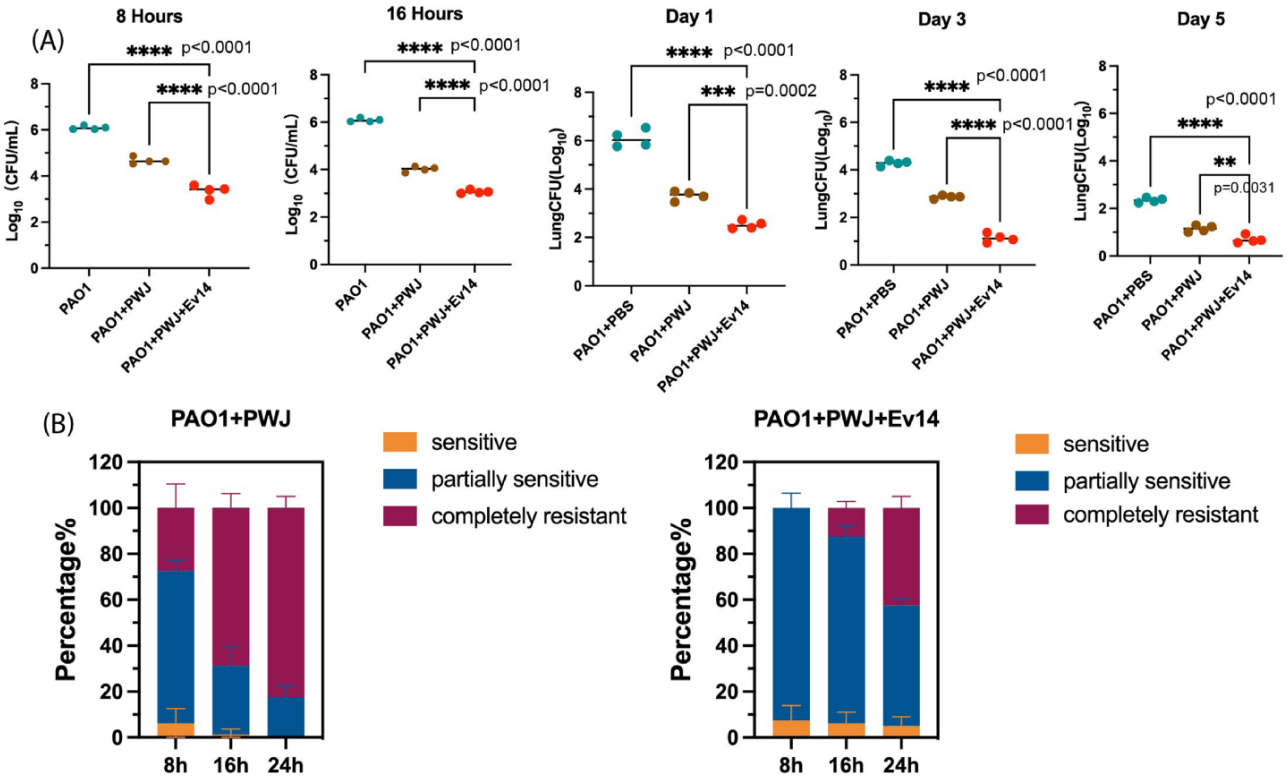

Figure 7. Therapeutic efficacy of phage PWJ alone and joint application (PWJ +WJ_Ev14) in a mouse pneumonia model using PAO1. (A) Bacterial burdens were then determined at hour 8, hour 16, day 1, day 3, and day 5 and (B) ratios of sensitive, partially resistant, and completely resistant strains were also determined at hour 8, hour 16 and day 1. Significance was determined using one-way ANOVA (*$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, ns: no significance ($P > 0.05$)).

DNA and its large subunit has endonucleolytic and ATPase activities (Oliveira, Alonso, and Tavares 2005). This indicated that these phages may have unique characteristics in DNA packaging. Strikingly, we also observed that the representative vRNAP of these six phages had a closer evolutionary relationship with *Luzseptimavirus* LUZ7 rather than the phage of the same genus including YH6, probably implying diverse evolutionary trajectories between the two genera.

Evolved pre-adapted/trained phages are interesting alternatives to the parental phage (Rohde et al. 2018). In our study, a co-evolved training phage WJ_Ev14 that could re-lyse the phage-resistant bacterial strain PWJ^{M1} was successfully isolated. WJ_Ev14 was similar to PWJ in physical and chemical properties suggesting a continued therapeutic potential for clinical application. Interestingly, these phages differed by a single SNP that resulted in the R1051S mutation in the tail fiber protein. This most likely led to association with a new binding site on the PWJ-resister PWJ^{R1}. In a similar study, an evolved *Escherichia* phage HP3.1 displayed bacterial killing for most of the resisters compared to its ancestor HP3. A comparative genomic analysis of HP3.1 to HP3 indicated just two SNPs resulting in a KY464 to RH464 changes consistent with host binding and a missense mutation in the long tail fiber gene resulting in an N9R change in the spike protein (Salazar Keiko et al. 2021). Another study reported that a trained phage λtrn that was a descendant of phage λ could infect via both the LamB classical receptor as well as OmpF (Meyer et al. 2012; Borin et al. 2023) indicative of an expanded host range. These findings indicate that mutations in tail fibers presumably enhanced phage binding to bacterial resisters.

Our TEM observations and phage absorption assays confirmed that the cell surface structure of the bacterial resister strain PWJ^{M1} changed from smooth to rough implying a cell surface alteration. Furthermore, phage absorption assays indicated that PWJ and WJ_Ev14 could both efficiently adsorb to ATCC 27853 whereas PWJ did not absorb to the resister PWJ^{R1}. Phage adsorption to cell cognate receptors is the initial step of infection and some bacterial strains have developed mechanisms to escape under phage selective pressure (Wang et al. 2023a). The results in our study suggest that phage WJ_Ev14 might be able to re-infect PWJ^{M1} resisters through compensatory mutations and is related to the R1051S mutation we identified.

Bacteria can evolve resistance and prevent virus attack under strong selective pressures of lytic phages. The emergence and enrichment of phage-resistant genotypes might result in therapy failure (Singh et al. 2023). Hence, the use of combinations of pre-adapted and ancestral phage as therapeutics has been demonstrated to mitigate the evolution of phage resistance (Laanto et al. 2020; Eskenazi et al. 2022). In this study, PWJR1 probably had evolved to a state with low fitness cost during five transfers over 10 d and the training phage WJ_Ev14 restored infectivity to PWJ^{R1}. We then hypothesized that host evolution to further resistance would be more difficult under the dual pressure of PWJ and WJ_Ev14. Like evolutionary trajectories of antibiotic resistance, evidence had stated that the fate of a resistance mutation in pathogen populations is determined in part by its fitness. Mutations that suffer little or no fitness cost are more likely to persist in the absence of antibiotic treatment (Melnyk, Wong, and Kassen 2015).

On the other hand, antimicrobials target essential cellular functions but bacteria could become resistant by acquiring either exogenous genes or chromosomal mutations. Resistance mutations typically occur in genes encoding essential functions; these mutations are therefore generally detrimental in the absence of drugs. However, bacteria can reduce this handicap by acquiring additional mutations, known as compensatory mutations and into a state of high adaptability (Durão, Balbontín, and Gordo 2018). Thus, the combination cocktail could stop the host bacteria to the most adaptive state of evolution, forcing an increase in the fitness cost and improvement in the phage therapy efficacy. Indeed, our findings were consistent with expectations and WJ_Ev14 lysed both ATCC 27853 and PAO1 more efficiently than the ancestral PWJ. This also resulted in prolonged suppression of resistant host populations. These findings indicated that coevolutionary phage training is a promising strategy to improve therapeutic efficacy in phage therapy.

Reports of the successful use of evolved phage have also been documented in different bacterial hosts. For example, a cocktail of ancestral phage phiYY and trained phage PaoP5-m1 with 3 broad host range *P. aeruginosa* phage was effective against diverse collection of *P. aeruginosa* clinical isolates and in the short-term, constrained the appearance of the phage-resistant mutants (Yang et al. 2020b). Additionally, the trained phage P3-CHA coevolved from PAK_P3 in *P. aeruginosa* significantly increased therapeutic efficiency *in vitro*. Strikingly, these results were confirmed *in vivo* in a mouse lung infection model and increased mouse survival from 20 to 100 per cent (Morello et al. 2011). In another study, four evolved *P. aeruginosa* phages were more efficient in reducing bacterial densities than ancestral phages (Friman et al. 2016) and a group of four different evolved phage reduced ancestral host population sizes of *P. aeruginosa* (Betts et al. 2013).These results have also been extended outside the clinic and a trained *P. aeruginosa* phage FCV-1.01 selected from a co-culture with myophage FCV-1 with its host the fish pathogen *Flavobacterium columnare* was able to infect 18/32 resisters (Laanto et al. 2020). These results as well as our findings implied that evolutionary training of phages could be employed as an effective tool of combatting bacterial infections and that artificial selection of a therapeutic phage by phage training can be utilized.

It is worth noting that the current accessibility to phage therapy from the lab bench to the bedside is still extremely low (Hesse and Adhya 2019). Phage therapy trials are underway in the areas of (i) medical tourism to establish phage therapy clinics, (ii) compassionate-use programs, and (iii) clinical trials (Altamirano and Barra 2019). The problems as indicated above still present an obstacle to a successful therapy (Rohde et al. 2018). For instance, evolved phage with potential therapeutic value is isolated from different time points during the co-evolution process and this will probably imply a limitation for their future clinical application. Usually in intensive care units where patients need to be cured within minutes or hours, implementation of a well-defined trained phage would be difficult due to time limitations because phage training protocols usually require a week or more time to be performed (Merabishvili, Pirnay, and De Vos 2018). Nevertheless, there are several measures that could still be used to avoid deficiencies: (i) a 'one-size-fits-all' strategy by training phages on already available representatives of local critical strains and (ii) descriptions of the evolution trajectories of phage resisters to know 'what happened' and also understanding of 'how' evolution of trained phage has proceeded in regaining infectivity to the resister (Baquero et al. 2021). Therefore, trained phage could be an important part of the human arsenal in combating AMR pathogens.

5. Conclusions

In summary, an N4-like co-evolved training phage WJ_Ev14 that could regain infectivity to the resistant bacteria was isolated in this study. The combination of WJ_Ev14 and its ancestral phage

PWJ led to both improved killing efficiency and prolonged suppression of host populations *in vitro* and *in vivo*. This represents a promising candidate for inclusion in phage cocktails developed for *P. aeruginosa* infections. Further identification of re-lysis mechanisms and the involvement of the R1051S mutation in the tail fiber protein may lead to a new binding site on the PWJ-resister PWJ^{R1}. This implies that point-directed phage tail fiber editing of this target mutation may expand the lysis spectrum. Multiply-drug resistant pathogens have renewed interest in phage therapy but current accessibility to phage therapy in clinics is still extremely low. Implementation of a well-defined trained phage would also be difficult due to time limitations. A 'one-size-fits-all' strategy by training phages on representatives of strains will be useful for phage bank construction. Future studies of the evolution pathways and trajectories of phage resisters to expand knowledge regarding how evolution of trained phage occurred will provide a more comprehensive understanding of the mechanisms involved in the evolution of phage resistance. Phage training can lead to improved treatment outcomes and pre-adaptation to the phage-resistant bacterial populations during co-evolution using phage training will still be of great value in guiding the designation of better phage and as important tools for precision phage engineering.

Supplementary data

Supplementary data is available at *Virus Evolution* online.

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