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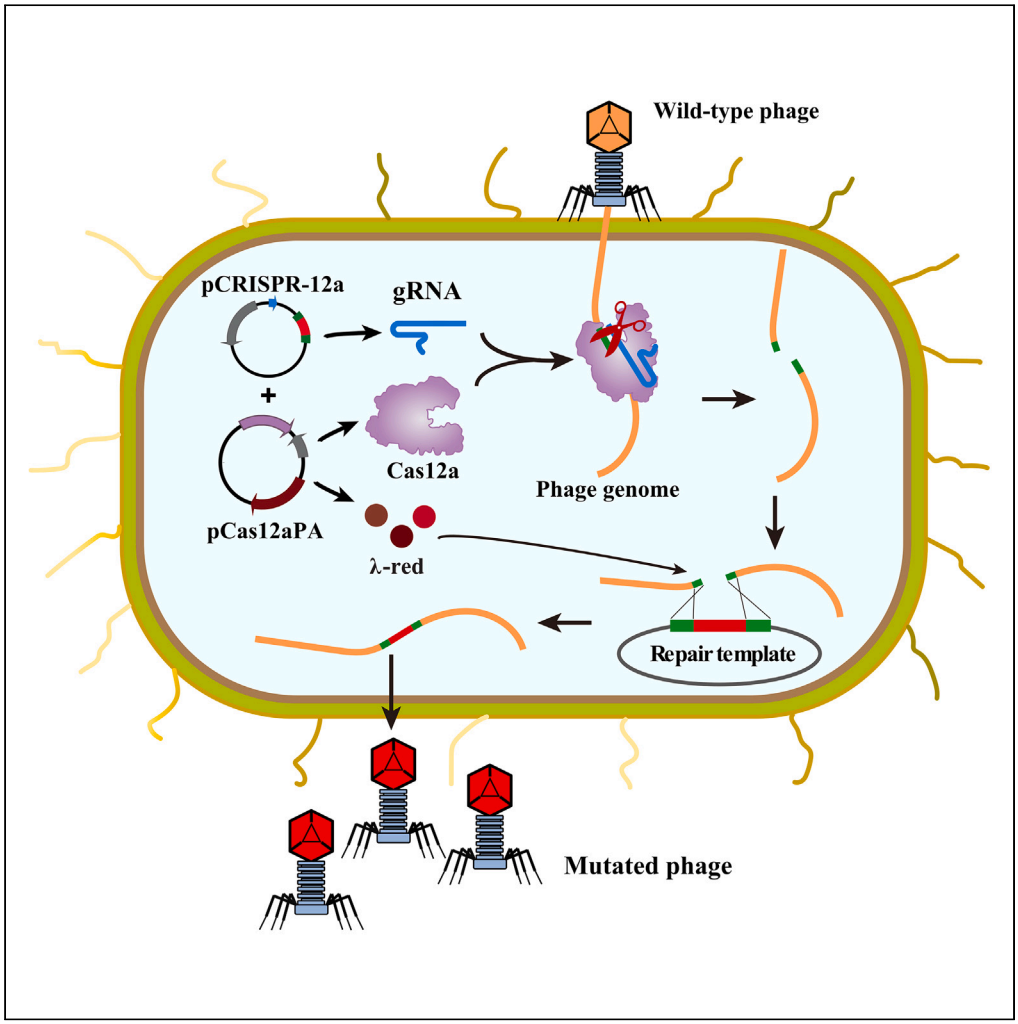

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Highlights

P. aeruginosa phages edited via CRISPR-Cas12a successfully

The CRISPR-Cas12a outperforms CRISPR-Cas9 in cutting genes

This study has achieved precise gene editing in multiple phage types

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Development of the CRISPR-Cas12a system for editing of *Pseudomonas aeruginosa* phages

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SUMMARY

Pseudomonas aeruginosa is a common opportunistic pathogen. The potential efficacy of phage therapy has attracted the attention of researchers, but efficient gene-editing tools are lacking, limiting the study of their biological properties. Here, we designed a type V CRISPR-Cas12a system for the gene editing of _P. aeruginosa_ phages. We first evaluated the active cutting function of the CRISPR-Cas12a system _in vitro_ and discovered that it had a higher gene-cutting efficiency than the type II CRISPR-Cas9 system in three different _P. aeruginosa_ phages. We also demonstrated the system's ability to precisely edit genes in _Escherichia coli_ phages, _Salmonella_ phages, and _P. aeruginosa_ phages. Using the aforementioned strategies, non-essential _P. aeruginosa_ phage genes can be efficiently deleted, resulting in a reduction of up to 5,215 bp (7.05%). Our study has provided a rapid, efficient, and time-saving tool that accelerates progress in phage engineering.

INTRODUCTION

Pseudomonas aeruginosa is one of the most common opportunistic nosocomial pathogens and can cause a range of infections, including pneumonia, urinary tract, and skin infections.[1] However, the increasing resistance of this bacterium to antibiotics has become a significant clinical concern.[2] In particular, the emergence of carbapenem-resistant *P. aeruginosa* complicates antibiotic treatment.[3]

Bacteriophages (phages) are viruses that specialize in infecting host bacteria and are responsible for controlling the number of pathogens in the environment.[4] They have the ability to lyse (destroy) host cells, which helps control the spread of bacteria.[4] Phages do this by injecting their own DNA into a host cell, which then takes over the cell's replication machinery and produces more phage particles, eventually causing cell lysis and death. *P. aeruginosa* phages have been used in a variety of applications, including the biocontrol of *P. aeruginosa* in hospitals[5] and food processing plants,[6] the bioremediation of *P. aeruginosa*-contaminated environments,[7] and the treatment of humans and animals for *P. aeruginosa* bacterial infections.[8–10]

Fortunately, the recent development of CRISPR-Cas system provided an efficient and convenient tool for gene editing.[11,12] The type II CRISPR-Cas9 system is the most commonly used CRISPR system, which consists of a Cas9 nuclease and a single guide RNA (gRNA).[13–15] CRISPR-Cas9 can be easily programmed to target a specific sequence by providing a gRNA that is complementary to the desired DNA sequence. Once bound to the target sequence, the Cas9 nuclease cleaves both DNA strands, resulting in a double-strand break. Type V CRISPR-Cas12a, also known as Cpf1, is another CRISPR-Cas system that has recently gained popularity due to its applications in gene editing.[16] Compared with CRISPR-Cas9, CRISPR-Cas12a offers numerous advantages. For instance, the Cas12a protein is smaller than Cas9, making it easier to package into viral vectors for delivery to cells.[17] Additionally, it has a more streamlined RNA guide sequence that is easier to design and program.[18,19] Furthermore, CRISPR-Cas12a exhibits a lower risk of off-target effects than Cas9, allowing for more precise gene editing.[18,19]

Numerous researchers have focused on the gene editing of *P. aeruginosa*. For instance, phage-encoded homologous recombination (PEHR) is an effective tool for bacterial genome engineering, and Zheng et al. developed a *Pseudomonas*-specific PEHR-Cas3 system. In contrast to traditional allelic-exchange editing methods, which require the construction of suicide plasmids carrying long homology arms, the PEHR-Cas3 system streamlines the experimental process by simplifying the required components and shortening the traceless editing cycle.[20] Lin et al. demonstrated that the CRISPR-Cas12a system is an effective editing system that can delete 15 kb from the genome of *P. aeruginosa*.[21] Sun et al. established an efficient cytosine-base-editing system using cytidine deaminase, enhanced-specificity Cas9 nickase,

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and a uracil DNA glycosylase inhibitor for use in *Pseudomonas* species.[22] Our previous work has also shown that the CRISPR-Cas9 system can accurately delete and insert genes in *P. aeruginosa*.[23,24] However, little research has been undertaken on the genome editing of *P. aeruginosa* phages.

In this study, we established a type V CRISPR-Cas12a phage-editing system for this purpose. Our results demonstrated that the CRISPR-Cas12a system exhibited more efficient cleavage activity than CRISPR-Cas9 systems in three different *P. aeruginosa* phage species (PJNP013, PJNP029, and PJNP053). Furthermore, the CRISPR-Cas12a system efficiently introduced point mutations, deletions, and insertions into phage genomes. Our study has introduced a rapid, efficient, and time-saving tool that accelerates the progress in *P. aeruginosa* phage engineering.

RESULTS AND DISCUSSION

Engineering type V CRISPR-Cas12a systems for *in vitro* verification of active cutting functionality

Research has shown that type V CRISPR-Cas12a systems have greater cleavage activity against *E. coli* phage genomes than type II CRISPR-Cas9 systems.[25] To our knowledge, these two systems have rarely been used for the gene editing of *P. aeruginosa* phages. However, it remained to be determined whether these two systems have similar cleavage activities in *P. aeruginosa* phages. To develop the type V CRISPR-Cas12a system for the genome editing of *P. aeruginosa* phages, we constructed a two-plasmid system, with a pCas12aPA plasmid expressing Cas12a from *Francisella novicida* U112 (FnCas12a) and a pCRISPR-12a plasmid expressing the corresponding gRNA (Figure S1). Then, we verified the cleavage efficiency of the CRISPR-Cas12a system on *P. aeruginosa* genomes while using the CRISPR-Cas9 system on a control group.[24] The results showed that there was no bacterial growth on any of the tested plates (Figure S2A). No differences between the CRISPR-Cas12a and CRISPR-Cas9 systems were observed for the same cleavage site (Figure S2B). Our results demonstrated that the established CRISPR-Cas12a system has effective cleavage activity against the *P. aeruginosa* genome.

Superior gene-cutting efficiency of the type V CRISPR-Cas12a system compared to the type II CRISPR-Cas9 system for *P. aeruginosa* phage genomes

To determine whether the type II CRISPR-Cas9 and type V CRISPR-Cas12a systems have efficient cleavage activity against *P. aeruginosa* phage genomes, three *P. aeruginosa* phages (PJNP013, PJNP029, and PJNP053) were selected for gene editing. These three phages were all isolated from hospital sewage using the double-layer culture method, with *P. aeruginosa* YP25-2 as the host bacteria. Electron microscopy analysis revealed that PJNP029 had a long tail with an isometric polyhedral head, while PJNP013 and PJNP053 had short tails with isometric polyhedral heads (Figure 1A). The co-culture assay demonstrated the bacteriostatic or bactericidal abilities of the three phages (PJNP013, PJNP029, and PJNP053) against their common host bacteria YP25-2 (Figure 1B).

Genetic analysis further showed that the genome of PJNP013 had 93% nucleotide identity (93% coverage) with phage LKD16 (GenBank no. NC_009935), which belongs to the Autographiviridae family; PJNP029 had 99% nucleotide identity (98% coverage) with phage phipa10 (GenBank no. NC_073620), which belongs to the *Pakpunavirus* genus; and PJNP053 had 89% nucleotide identity (93% coverage) with phage LUZ7 (GenBank no. NC_013691), which belongs to the Schitoviridae family (Figures S3 and S4). The amino acid sequences of the terminase large subunit proteins from the three phages suggested that PJNP013, PJNP029, and PJNP053 belonged to the *Phikmvvirus*, *Pakpunavirus*, and *Luzseptimavirus* genera, respectively (Figures 1C and S3).

To test the cleavage effects of CRISPR-Cas9 and CRISPR-Cas12a systems on *P. aeruginosa* phages, we randomly selected 5 to 9 target sites from the genomes of the three phages (PJNP013, PJNP029, and PJNP053) and separately assembled each of these spacer sequences into editing plasmids (Figure S5). Then, we transformed *P. aeruginosa* with the editing plasmids and assessed the phage-gene-cleavage activity. For phage PJNP029, the antiviral activity of each spacer in the CRISPR-Cas9 system varied, with a plaque formation efficiency of $\sim10^{-1}$ to 10^{-3}. In contrast, in the CRISPR-Cas12a system, each spacer showed a higher cutting efficiency and a plating efficiency of $\sim10^{-2}$ to 10^{-6} (Figure S5). For phage PJNP013, a plating efficiency of $\sim10^{-1}$ was observed with the CRISPR-Cas9 system, lower than the plating efficiency (10^{-1} to 10^{-4}) observed with the CRISPR-Cas12a system (Figure S5). For phage PJNP053, none of the spacers induced an immune response with the CRISPR-Cas9 system, but each spacer showed different levels of antiviral activity, with plaque formation efficiencies of $\sim10^{-1}$ to 10^{-4}, with the CRISPR-Cas12a system (Figure S5). There were no obvious correlations between the GC or C contents of the protospacer sequences and the gene-cutting efficiency (Figure S5; Table S1). The discrepancies in antiviral immunity observed for the different phages may have been related to the spacers used in the CRISPR-Cas12a system; for example, phage PJNP053 gRNA-S1 and S13 and phage PJNP013 gRNA-S1 and S6 showed limited cutting efficiencies (10^{-1} to 10^{-2}) in the host cell, while phage PJNP013 gRNA-S3 and S4 and phage PJNP053 gRNA-S2, S4, S6, S7, and S11 spacers showed higher cutting efficiencies and plating efficiencies of 10^{-4} to 10^{-6}.

Type II and type V CRISPR systems have distinct cleavage site targets and methods. For instance, the FnCas12a nuclease cleaves 23 nucleotides of the protospacer and creates a 5-nt sticky end,[18] while the SpCas9 system cleaves 3-bp upstream of the protospacer adjacent motif (PAM) site, resulting in blunt ends.[26] To further investigate whether the CRISPR-Cas9 and CRISPR-Cas12a systems exhibit different cleavage activities toward the same target site, we selected overlapping target sites that could be recognized by both CRISPR-Cas9 and CRISPR-Cas12a systems and assembled them into editing plasmids (Figure 2). Subsequently, phage PJNP029 was used to infect *P. aeruginosa* YP25-2 cells containing either CRISPR-Cas12a or CRISPR-Cas9 plasmids. The efficiency of plating (EOP) values was then calculated. The results showed that the EOP of PJNP029 in *P. aeruginosa* YP25-2 cells containing the CRISPR-Cas12a system was 10 to 10^4 times higher than that of the CRISPR-Cas9 system (Figure 2). These findings indicate that the CRISPR-Cas12a system has stronger cleavage ability toward the same target sites in phage genomes than the CRISPR-Cas9 system. Therefore, we chose the CRISPR-Cas12a system for subsequent experiments.

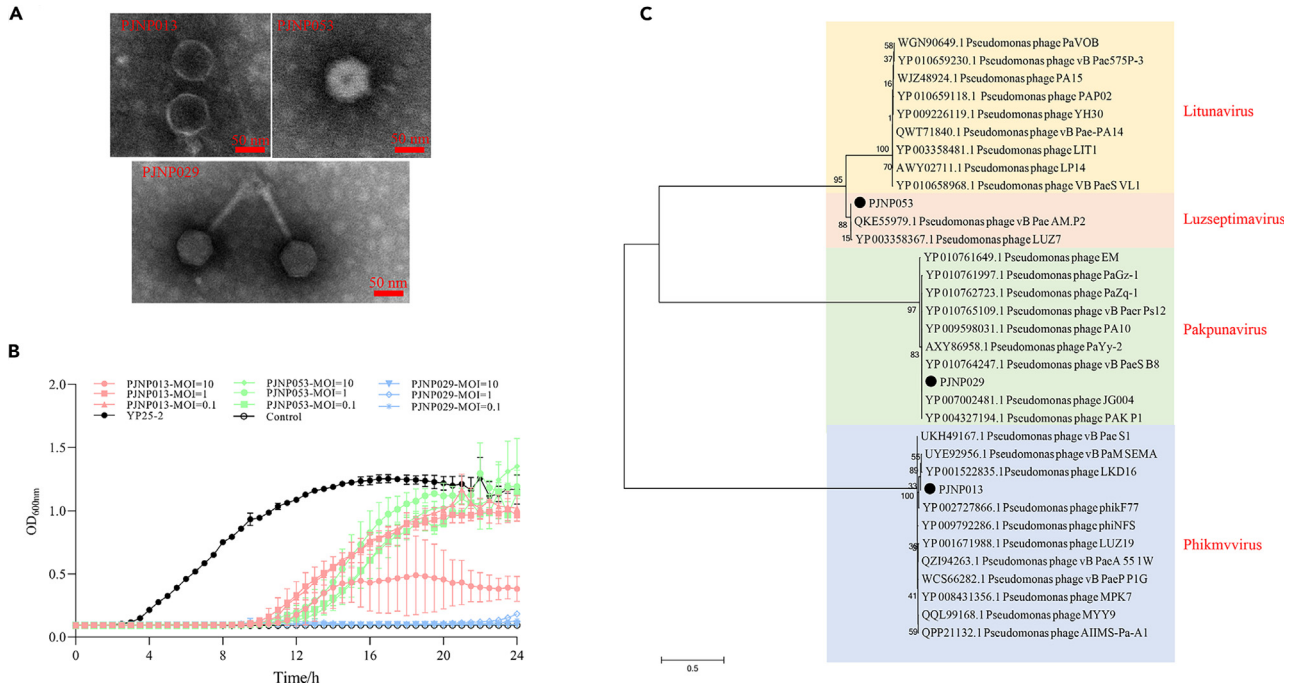

Figure 1. Biological characteristic and phylogenetic tree analysis of phages PJNP013, PJNP029, and PJNP053

(A) Transmission electron micrograph. Bar, 50 nm.

(B) Effect of three phages on bacterial growth of host strain YP25-2. This experiment was repeated three times and the data are expressed as the mean ± SD.

(C) Phylogenetic tree analysis of the phage-encoded large terminase subunit. The phylogenetic tree was generated using the neighbor-joining method and 1,000 bootstrap replicates.

Research has shown that the number of specific spacers in the bacterial CRISPR system is positively correlated with the ability of the CRISPR system to resist phage infection.[25,27] Therefore, we inserted two spacers into an editing plasmid simultaneously and assessed the effect of the CRISPR-Cas12a system containing two spacer sequences on phage infection. The results showed that the efficiency of the double-spacer CRISPR-Cas12a system in resisting phage infection was significantly higher than that of the single-spacer CRISPR-Cas12a system ($p < 0.05$) (Figure 3).

Evolution mechanism of mutant phage escape under the pressure of type V CRISPR-Cas12a systems

We noticed that some phages survived under CRISPR-Cas12a selective pressure. To clarify the mechanism by which the phages evaded the Cas12a nuclease attack, we conducted a preliminary analysis of the protospacer and PAM regions of PJNP029 "CRISPR-escaping" phages using Sanger sequencing. Only one base mutation in the protospacers of all 10 randomly selected mutants was detected in the first-generation plaques with the high-efficiency spacer (gRNA-S3 with plating efficiency of $\sim 10^{-5}$) (Figure 4A). This suggested that phage PJNP029 employed a complex mechanism to escape Cas12a cleavage. With the constant selective pressure of the CRISPR-Cas12a system, rapid evolution and selection of mutants were observed. By the fifth generation, mutations were observed in 9 out of 10 protospacer sequences (Figure 4A).

The battle for survival between phages and bacteria may drive the evolution of both organisms. We further evaluated the lytic ability of mutated phages against the host bacteria. The plaque formation assays revealed that the lysis ability of two (#3 and #5) of the nine mutant phages tested was not significantly different from the wild-type phages (Figure 4B). The results of co-cultivation tests indicated that phage mutants #3 and #5 induced no obvious growth changes compared to the wild-type PJNP029 (Figure 4C). Under the action of the low-efficiency FnCas12a-gRNA complex (gRNA-S1 with plating efficiency of $\sim 10^{-1}$), the CRISPR-escaping phage had a minimal effect on the protospacer and PAM sequences. For instance, in the first generation of 10 randomly selected phages, the protospacer and PAM sequences had no mutations, while under continuous pressure, only 20% of the fifth-generation phages had protospacer sequence changes (data not shown). Our findings suggest that phages participate in a complex mechanism to evade Cas12a nuclease attack under CRISPR-Cas12a selective pressure.

CRISPR-Cas12a system for efficient gene deletions, point mutations, and gene insertions in phage genomes

Gene point mutations, gene deletions, and insertions of phages have potential applications in phage therapy. For instance, knocking out phages can reduce the risk of the spread of phages carrying resistance genes or virulence genes. Therefore, we tried to harness the CRISPR-Cas12a system for the genetic manipulation of phage genomes. We first selected the relatively high- (gRNA-S4 with plating efficiency

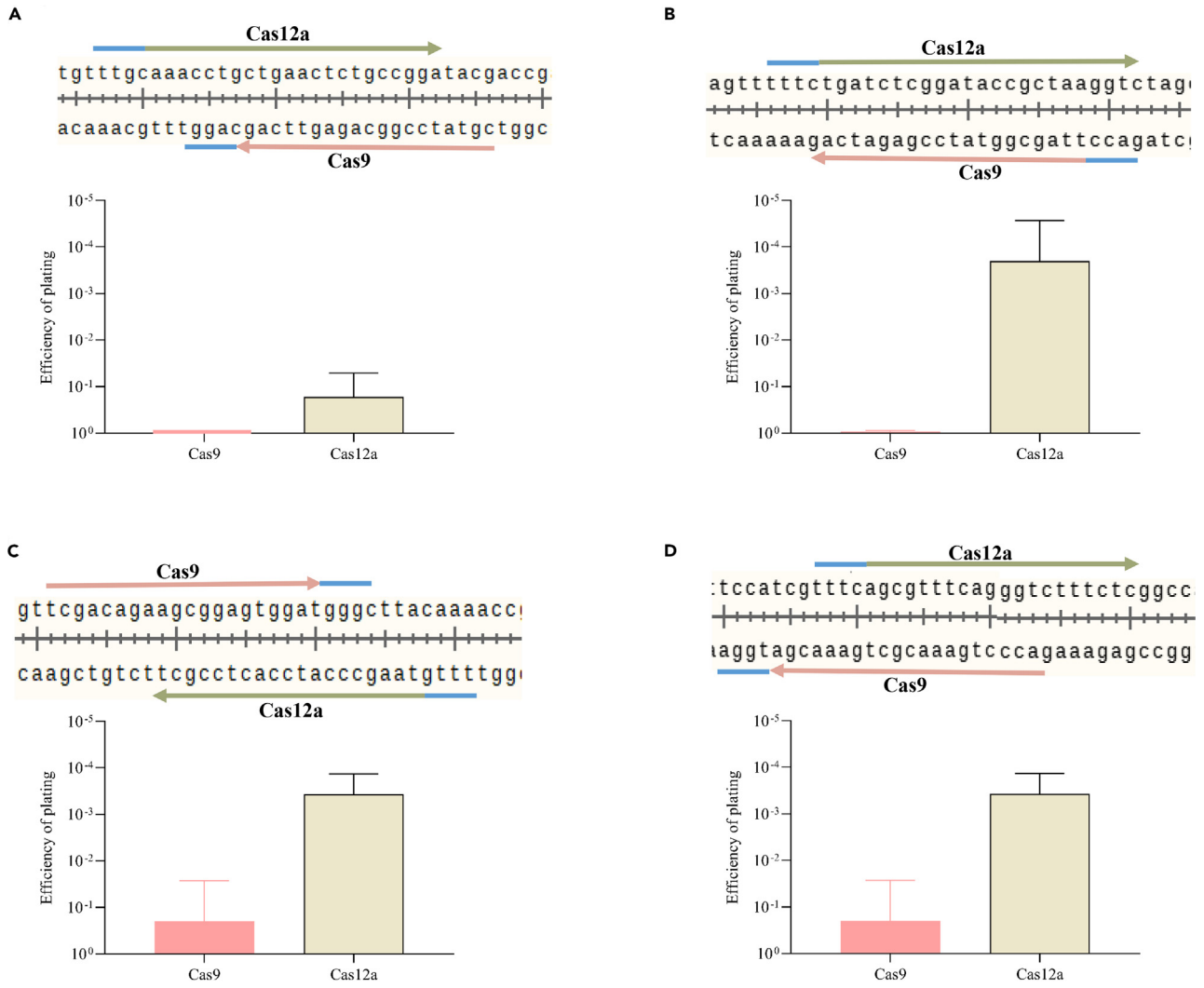

Figure 2. Verification of the cleavage efficiency of the *P. aeruginosa* phage genomes

Figure (A–D) indicated 4 different overlapping cleavage sites. This experiment was repeated three times and the data are expressed as the mean ± SD.

of ~10^{-4}, targeting the RNA polymerase) and weak- (gRNA-S1 with plating efficiency of ~10^{-1}, targeting the DNA polymerase) activity gRNAs from phage PJNP053 and assembled the homology arms, with a length of 500 bp, into the editing plasmids to facilitate homologous recombination repair after DNA double-strand break. The results of Sanger sequencing and PCR revealed that 505 bp of the RNA polymerase gene was successfully deleted and tiny phage plaques formed on the plates (Figures 5A and S6A). In addition, 538 bp of the phage DNA polymerase gene targeted by the weak gRNA-S1 was also successfully deleted. PCR amplification of possible phage mutants revealed 100% (n = 10) positive clones, and pinpoint-shaped plaques were observed on the plate (Figures 5B and S6B). Phage DNA and RNA polymerases are key enzymes responsible for replication and transcription in phages, and a lack of DNA polymerase can affect phage survival.[28] The phenomenon of mutated phages has also been observed in other phage-genome-editing applications.[29]

Further analysis of the ability of the CRISPR-Cas12a system to induce phage point mutations showed that an editing efficiency of 100% (n = 10) could be obtained (Figure 6A). Sanger sequencing of the PCR amplification products confirmed base editing was successful (Figure 6A).

To assess the ability of CRISPR-Cas12a-mediated genome editing of the lytic PJNP029 phage, we inserted a 699-bp eGFP gene fused with homology arms of 500 bp. PCR amplification of the possible recombinant PJNP029 phage showed 100% (n = 10) positive clones (Figure 6B). Sanger sequencing of the PCR amplification products confirmed the success of the base editing (Figure 6B). To verify the stability of the recombinant eGFP phage, we performed continuous blind passaging of the recombinant phage for 20 generations. PCR showed that the recombinant phage did not undergo eGFP gene loss. We further analyzed the insertion efficiencies using plasmids with different homology arm lengths, from 30 to 500 bp. The results showed that an editing efficiency of 100% (n = 10) was obtained with homology arm lengths of 100 to

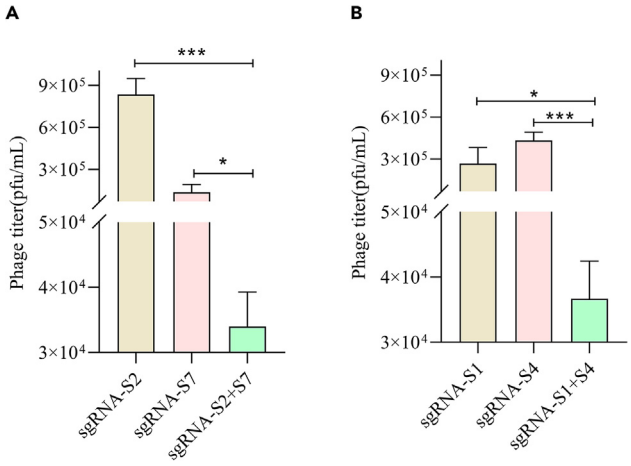

Figure 3. Efficiency of plating of phages under single or two different FnCas12a-gRNA complex pressure

(A) PJNP029 and (B) PJNP053.

This experiment was repeated three times and the data are expressed as the mean ± SD. The significant differences were indicated by *$p < 0.05$ and ***$p < 0.001$.

500 bp (Figure 6C). Unfortunately, no recombinant phages were obtained when the homology arms were 30 to 50 bp in length. We speculated that this may have been due to the lambda-Red recombination efficiency (Figure 6C).

We further employed the CRISPR-Cas12a gene editing system to assess its gene-cutting ability on *E. coli* phages (T4 and T7) and *Salmonella* phages (PJNS016 and vB_SalS_JNS02). The results demonstrated that this system exhibited varying cutting efficiencies at different target sites for these phages (Figure S7). Subsequently, gene knockout procedures were performed in *Salmonella* phage vB_SalS_JNS02 as described earlier (gRNA-S3 with plating efficiency of $\sim 10^{-1}$, targeting the head morphogenesis) (Figure S8A). Through PCR analysis, we successfully obtained 100% ($n = 10$) potentially positive clones (Figure S8B). Additionally, Sanger sequencing further confirmed the successful deletion of the target genes (Figure S8C).

Streamlining phage genomes: Removing non-essential genes to create genetic space

Tail phages typically have relatively large (ranging from 4 to 735 kb) and extremely diverse genomes.[30,31] In addition to the genes necessary for maintaining life and adapting to the host, the phage may carry other non-functional genes that occupy a significant portion of the phage genome. For instance, non-essential genes may be present that assist the phage in adapting to various ecological niches.[32] Under certain conditions, these genes are unnecessary for phage development. Therefore, space can be created by removing non-essential genes to better utilize phages to create recombinant vaccines or carry drug-targeting molecules.[33] Moreover, streamlining the non-essential genes of phages can improve phage bactericidal activity.[34] Recently, Yuan and colleagues developed a CRISPR-Cas9-based iterative phage-genome-reduction approach that reduced the genome of four different tailed phages (T7, T4, seszw, and selz) by 8%–23%, and the mutated phages displayed different sensitivities to host bacteria. This suggested that a streamlined phage can serve as a biological chassis for future technological needs. Therefore, we established a rapid method to streamline phage genomes using the V CRISPR-Cas12a system.

First, we divided the putative non-functional gene sequences of PJNP053 into four main regions (Figure 7). Corresponding gRNA and homology arms were constructed for each functional region, and the editing plasmids were transformed into *P. aeruginosa* YP25-2. Then, phage PJNP053 was used to infect host cells containing the editing plasmids. Surviving phages indicated that the deletion of the region had no effect on the survival of the phage. The results of phage infection showed that, when only the D region of phage PJNP053 was deleted, no phage plaques were observed on the double-layer plate (Figures 7 and S9). We speculated that the D region may be an important functional gene of phage PJNP053. In contrast, deletion of the other three regions (A–C) had no impact on the survival of the phage (Figures 7 and S9). We further reduced the number of non-essential genes to create genetic space, and when the AB and AC combination regions were both deleted, the phage survived. However, when the BC and ABC combination regions were deleted, no plaques were observed on the plate, suggesting that the simultaneous deletion of the BC region negatively affected the biological activity of phage PJNP053. Our results revealed that using CRISPR-Cas12a gene editing technology can result in a reduction of the phage PJNP053 genome by 2,205–5,215 bp (representing 2.98%–7.05%, respectively) (Figures 7 and S9). We did not conduct an in-depth analysis of the impact of removing other non-essential genes of PJNP053 or phages PJNP013 and PJNP029, which will be further studied in the future.

We further compared the functions of the mutant phages with the smallest genomes with that of the wild-type phage in host bacteria. Co-culture results showed that the mutant phages with the smallest genomes and the wild-type phage PJNP053 were all able to inhibit the growth of the host bacteria YP25-2 for approximately 9 h (Figure 8A). The one-step growth curves of the phages showed that the wild-type PJNP053 had a burst size of 208 particles per cell in 60 min post-infection, while the particle release rate of the mutant phages with the smallest genomes decreased by varying degrees. For instance, PJNP053ΔA had a burst size of 103 particles per cell by 60 min

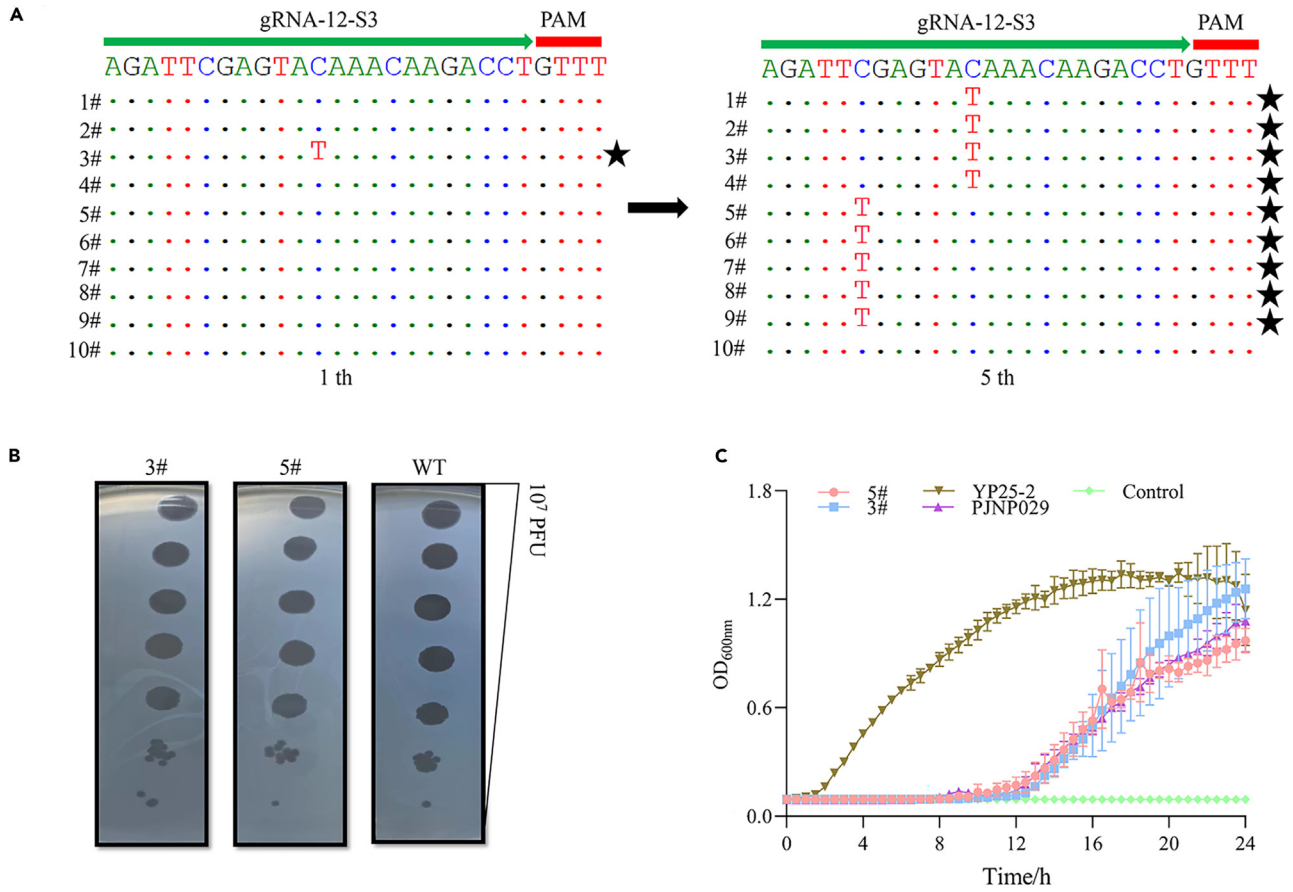

Figure 4. CRISPR-Cas12a pressure drives the evolution of escape mutant phages

(A) The escape phage's mutation pattern resulting from CRISPR-Cas9 pressure. 10 plaques from each infection were randomly selected, and Sanger sequencing was conducted. The nucleotide sequence of each mutation in the protospacer or PAM region was aligned with the corresponding wild-type (WT) phage PJNP029 sequence.

(B) Plaque assay with WT-PJNP029 and its mutations. Phage lysates were serially diluted 10-fold and spotted onto bacterial lawns, incubated at 37°C for 12 h.

(C) Co-cultivation curve of WT- PJNP029 and its mutations (3# and 5#) with host YP25-2. This experiment was performed in triplicate and data are expressed as means ± SD.

post-infection, while several other mutant phages with the smallest genomes only had burst sizes of 21–42 particles per cell by 60 min post-infection (Figure 8B).

Ethics statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Conclusion

In this study, we successfully developed a type V CRISPR-Cas12a system for genome editing of *P. aeruginosa* phages. *In vitro* experiments confirmed that the editing efficiency of the CRISPR-Cas12a system surpassed that of type II CRISPR-Cas9 systems. The CRISPR-Cas12a system effectively carries out gene editing on *E. coli* phages, *Salmonella* phages, and *P. aeruginosa* phages. By using this system, we were able to reduce the number of non-essential genes in the phages, thereby increasing genetic space. In future work, we aim to employ this system to identify further non-essential genes in phages and gain a deeper understanding of the relationship between the phages and their host bacteria. Overall, the evidence shows we have developed a precise and efficient type V CRISPR-Cas12a system for manipulating phages.

Limitations of the study

Although our research indicates that the type V CRISPR-Cas12a system demonstrates higher gene editing efficiency in *P. aeruginosa* phage compared to the type II CRISPR-Cas9 system, it is worth noting that different spacers may affect the cutting efficiency. In addition, we

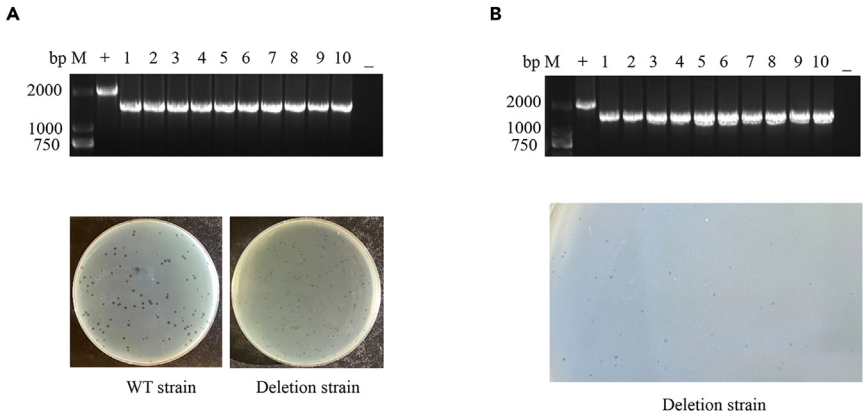

Figure 5. The CRISPR-Cas12a system mediates efficient gene deletions in phage genome

(A) Amplified PCR product. A pinpoint-shaped plaque was observed on the plate compared to WT strain. "+" represents the WT phage, numbers 1–10 designate potential recombinant phages, and "-" denotes the negative control.

(B) Amplified PCR product. A pinpoint-shaped plaque was observed on the plate compared to WT strain. "+" represents the WT phage, numbers 1–10 designate potential recombinant phages, and "-" denotes the negative control.

observed that the type II CRISPR-Cas9 system barely exhibited any cutting activity on the selected spacers of *P. aeruginosa* phage PJNP053. Whether this phage carries a certain anti-Cas9 protein remains uncertain and requires further investigation to verify.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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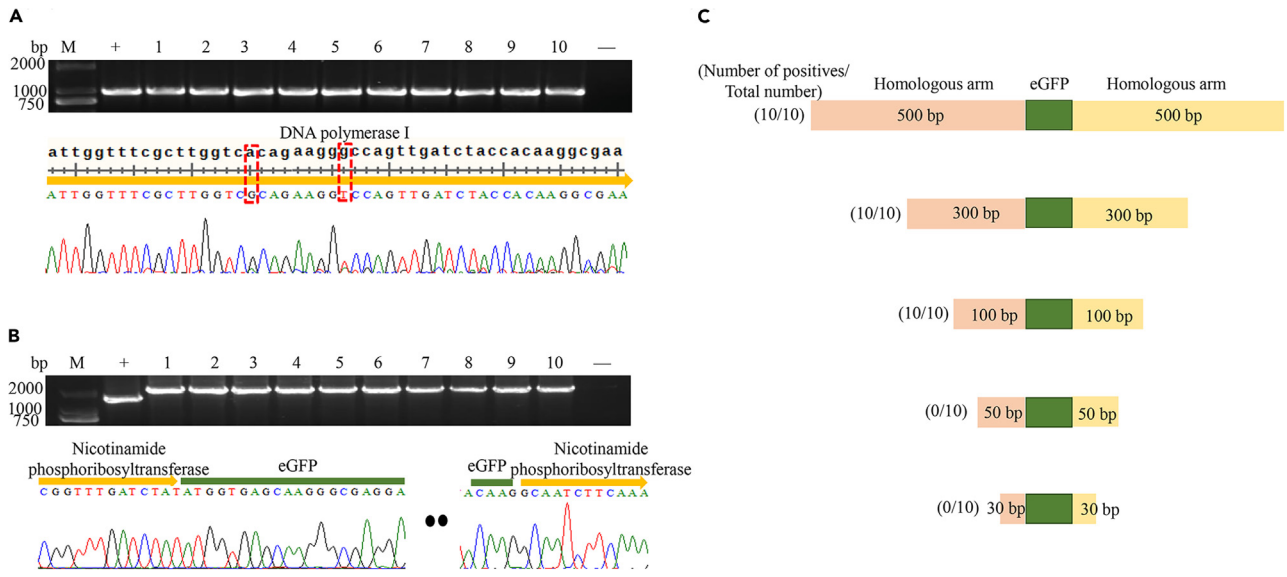

Figure 6. The CRISPR-Cas12a system enables efficient point mutations and gene insertions in phage genome

(A) Amplified PCR product and Sanger sequencing of the target gene. "+" represents the WT phage, numbers 1–10 designate potential recombinant phages, and "-" denotes the negative control.

(B) Amplified PCR product and detection of inserted reporter gene. "+" represents the WT phage, numbers 1–10 designate potential recombinant phages, and "-" denotes the negative control.

(C) An example of homologous arms for gene insertions.

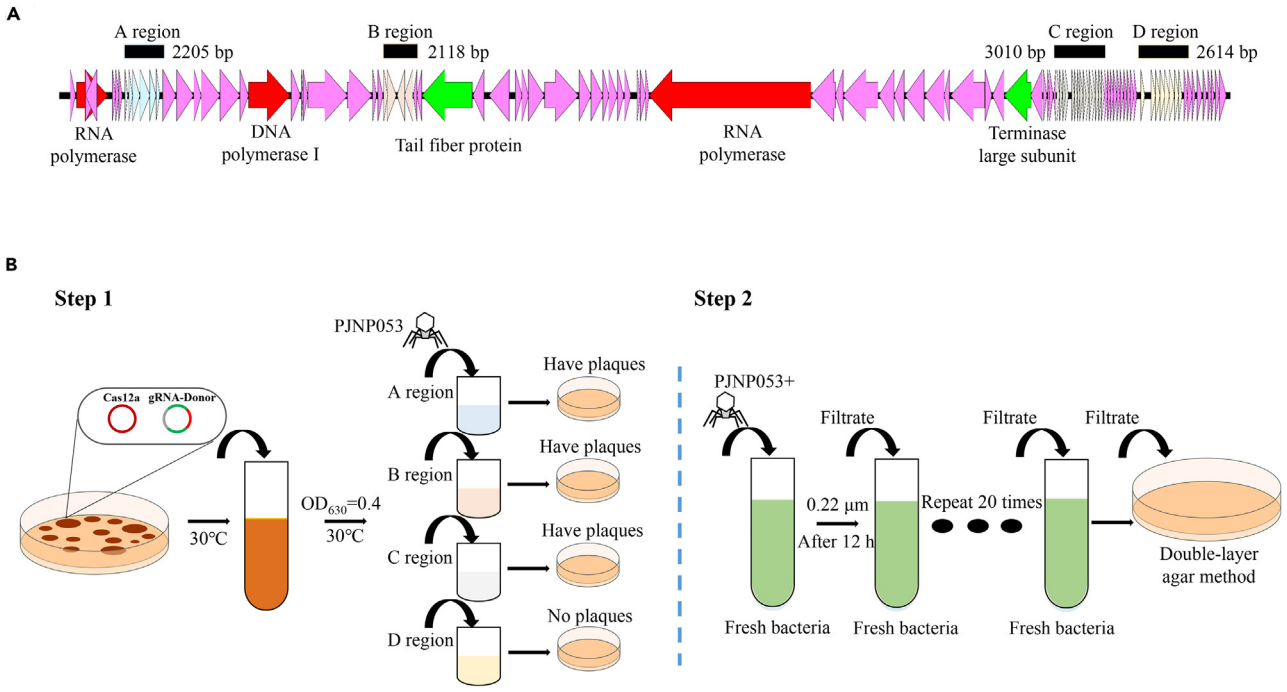

Figure 7. Schematic showing streamlining phage genomes

(A) Phage PJNP053 genome organization.

(B) Experimental scheme for reducing non-essential genes in phage PJNP053 genome.

- Materials availability
- Data and code availability

- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
- METHOD DETAILS

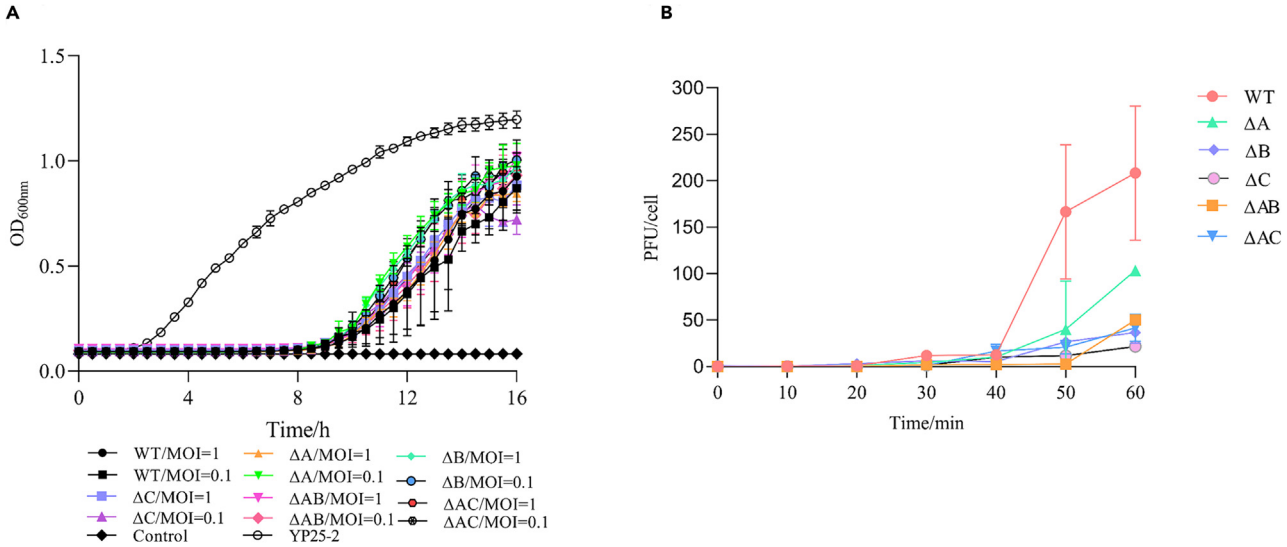

Figure 8. Phenotypic characteristics

(A) Effect of WT phage or mutant phages on bacterial growth.

(B) One-step growth curves of WT phage or mutant phages in host strain YP25-2. This experiment was repeated three times and the data are expressed as the mean ± SD.

- Bacterial strains, phages, and culture conditions
- Plasmid constructions
- pCRISPR-12a-gRNAXXX plasmid constructions
- Transformation assays
- Phage infection
- One-step growth curve

- QUANTIFICATION AND STATISTICAL ANALYSIS
- ADDITIONAL RESOURCES

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110210.

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AUTHOR CONTRIBUTIONS

Y. Chen drafted the main manuscript and performed the data analysis; B.Y., W.C., X. Zhang, Z.L., Q.Z., L.L., M.H., X. Zhao, X.X., Q.L., and Y. Luo planned and performed the experiments; and Y. Chen, Y. Cai, and Y. Liu were responsible for experimental design. All authors reviewed and agreed the publication of this manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
E. coli DH5α	Thermo Scientific™	CAS：EC0112
Salmonella	ATCC	ATCC14028
P. aeruginosa YP25-2	Laboratory Resource Library	N/A
P. aeruginosa phage PJNP013	Laboratory Resource Library	GenBank no. OR941786
P. aeruginosa phage PJNP029	Laboratory Resource Library	GenBank no. OR941787
P. aeruginosa phage PJNP053	Laboratory Resource Library	GenBank no. OR941788
Salmonella phage PJNS016	Laboratory Resource Library	GenBank no. PP723078
Salmonella phage vB_SalS_JNS02	Laboratory Resource Library	GenBank no. PP189839
Escherichia coli phage T4	Laboratory Resource Library	GenBank no. NC_000866
Escherichia coli phage T7	Laboratory Resource Library	GenBank no. NC_000866
Biological samples		
Hospital wastewater	Jinan Hospital	N/A
Chemicals, peptides, and recombinant proteins		
Cesium chloride	Huaxiangkejie	CAS：7647-17-8
Tetracycline	Shanghai kelamaer	CAS: 64-75-5
Carbenicillin antibiotics	Timentin	CAS: 4697-14-7/61177-45-5
L-arabinose	Solarbio	CAS:5328-37-0
Critical commercial assays		
TIANprep Mini Plasmid Kit	TIANGEN	DP103
Omega Original Adhesive R ecycling Kit 100t/box	Omega	D2500-50T
Phage genomic DNA Extraction Kit (PEG precipitation method)	ZiKER	ZK-L1938
Deposited data		
NCBI database	Genome Reference	https://www.ncbi.nlm.nih.gov/genome/
Phylogenetic Analysis for phage-encoded large terminase subunit.	MEGA	MEGA 8.0
Phylogenetic Analysis for phage genome	Viptree	https://www.genome.jp/viptree/upload
Statistical analysis	Graphpad Prism	Graphpad Prism 8.0
Oligonucleotides		
Primers targeting bacteriophages, see Table S1 and S5	This paper	N/A
Primers for recombinant phage, see Tables S2, S3, S4, and S5	This paper	N/A
Software and algorithms		
CRISPR	Web	http://chopchop.cbu.uib.no/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yibao Chen (yibaochen2012@126.com).

Materials availability

All unique/stable reagents in this study are available from the lead contact without restriction.

Data and code availability

- The complete genome sequence of phage PJNP013, PJNP029, PJNP053, PJNS016, and vB_SalS_JNS02, with annotation has been deposited in GenBank under accession number OR941786, OR941787, OR941788, PP723078, and PP189839, respectively
- This paper does not report original code.
- Additional information required to reanalyze the data in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

No experimental models were used in this study.

METHOD DETAILS

Bacterial strains, phages, and culture conditions

E. coli DH5α, *Salmonella* ATCC14028, and *P. aeruginosa* YP25-2 were cultured in LB broth at 37°C. If required, 100 μg/mL tetracycline (Thermo Scientific, China) and 150 μg/mL carbenicillin antibiotics (Thermo Scientific, China) were added to the medium. pCasPA (tetracycline-resistance gene, addgene plasmid #113347) and pACRISPR (carbenicillin-resistance gene, addgene plasmid #113348) were used in this study.

Three *P. aeruginosa* phages, PJNP013, PJNP029, and PJNP053, were isolated from hospital sewage using *P. aeruginosa* YP25-2 as the host bacteria. The phages were isolated using the conventional double-layer agar method as previously described.[35–37] The phage particles were then purified using CsCl (Sinopharm Group Chemical reagent Co., LTD, China) gradient ultracentrifugation and observed using a transmission electron microscope (JEM-1400Flash, Japan).[38] Co-cultures of host *P. aeruginosa* YP25-2 and phages or recombinant phages were incubated at 37°C in LB broth at different multiple of infection (MOI). The OD_{600} value was measured. This experiment was performed in triplicate, and the data are expressed as means ± SD.

Plasmid constructions

The construction of the pCas12aPA plasmid was carried out using the following approach. Initially, the gene encoding the Cas12a nuclease was amplified from the FnCas12a plasmid.[39] The purified amplicon was then cloned into the *Xho*I/*Hind*III (Thermo Scientific, China) sites of plasmid pCasPA, resulting in the recombinant plasmid pCas12aPA. Following the same protocol, the recombinant pCRISPR-12a plasmid was constructed by inserting the gRNA expression cassette into the *Nco*I/*Xba*I (Thermo Scientific, China) sites of the pACRISPR plasmid.

pCRISPR-12a-gRNAXXX plasmid constructions

The target gRNA sequences are designed using the web server CRISPR (http://chopchop.cbu.uib.no/). Two oligos were annealed and inserted into the pCRISPR-12a plasmid following the reaction procedure in Table S1. Recombinant pCRISPR-12a-gRNAXXX plasmids were transformed into *E. coli* DH5α using the heat shock transformation methods.[40]

Transformation assays

The pCas12aPA plasmids and pCRISPR-12a plasmids were mixed with 50 μL of competent bacteria (*E. coli* DH5α, *Salmonella* ATCC14028, and *P. aeruginosa* YP25-2). The mixture was then transferred to a 0.1-mm electroporation cuvette and electroporated with the parameters of 2100 V, 100 Ω, and 25 μF (Bio-Rad, US). After incubation on ice for 30 min, 100 μL of bacteria was added to coat an LB plate containing antibiotics (100 μg/mL tetracycline and 150 μg/mL carbenicillin) and incubated overnight at 37°C. Single colonies were selected, and L-arabinose (50 mmol/L) was added when the bacteria reached the growth phase (OD_{630} = 0.6), and these were incubated at 37°C for 2 h.

Phage infection

A 200-μL aliquot of host strains (containing pCas12aPA plasmids and pCRISPR-12a-gRNAXXX plasmids) was mixed with ~10^7 PFU of phages and poured onto 6 mL of soft LB agar containing 100 μg/mL tetracycline, 150 μg/mL carbenicillin, and 50 mmol/L L-arabinose (Shanghai Canspec Scientific Instruments Co., Ltd., China), and incubated at 37°C overnight. The conventional double-layer agar method was used to pick up ~10 random phage plaques. Genomic DNA was extracted from each purified possible recombinant phage as previously described.[41]

One-step growth curve

The one-step growth curve of phage was performed as described previously.[35,42,43] Briefly, wild-type phage or mutant phages with an MOI of 0.1 were incubated with 5 mL of exponentially growing bacteria YP25-2. The mixture was incubated at 37°C for 5 min, followed by centrifugation at 12,000 × *g* for 30 s. The pellets were then re-suspended in an equal volume of LB medium. At 0, 10, 20, 30, 40, 50, and 60 min, 100 μL of the mixture was taken and the phage titers were determined using the double-layer plate method. The burst size is calculated according to

the following formula: the final count of released phage progeny divided by the initial count of infected bacterial host cell during the latent period. This experiment was repeated at least three times.

QUANTIFICATION AND STATISTICAL ANALYSIS

The data was represented from the means ± standard deviation (SD) from three independent experiments.

ADDITIONAL RESOURCES

No additional resources are involved.