Chromosomal DNA deletion confers phage resistance to *Pseudomonas aeruginosa*

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Bacteria develop a broad range of phage resistance mechanisms, such as prevention of phage adsorption and CRISPR/Cas system, to survive phage predation. In this study, *Pseudomonas aeruginosa* PA1 strain was infected with lytic phage PaP1, and phage-resistant mutants were selected. A high percentage (~30%) of these mutants displayed red pigmentation phenotype (Red mutant). Through comparative genomic analysis, one Red mutant PA1r was found to have a 219.6 kb genomic fragment deletion, which contains two key genes *hmgA* and *galU* related to the observed phenotypes. Deletion of *hmgA* resulted in the accumulation of a red compound homogentisic acid; while a *galU* mutant is devoid of O-antigen, which is required for phage adsorption. Intriguingly, while the loss of *galU* conferred phage resistance, it significantly attenuated PA1r in a mouse infection experiment. Our study revealed a novel phage resistance mechanism via chromosomal DNA deletion in *P. aeruginosa*.

Bacteriophages and their bacterial hosts are constantly engaged in evolutionary competition1–3. On the one hand, bacteria employ a wide range of resistance mechanisms, including prevention of phage adsorption and phage DNA entry, digestion of phage nucleic acids, and development of abortive infection and CRISPR/Cas systems to evade phage infection or survive phage killing4–7. On the other hand, bacteriophages are capable of rapid adaptive responses to evolutionary changes in their hosts8. Adsorption of phages into bacterial hosts by specific interactions between phage proteins and receptors on the bacterial cell surface is the initial step of infection8,9. Several receptors, including lipopolysaccharides (LPS), capsular polysaccharides, flagella, and outer membrane proteins, have been reported5,8,9. As a defensive strategy, bacteria can modify these cell surface receptors and produce extracellular matrix or competitive inhibitors to prevent phage adsorption8. As a counter-defensive measure, phages are able to modify their receptor-binding proteins, such as tail fibers, to achieve infection and kill the resistant bacterium8,10. For example, *Pseudomonas fluorescens* SBW25 has co-evolved with its lytic phage phi2 for over 300 bacterial generations. This co-evolution is probably due to the continuous modification of bacterial receptors and phage receptor-binding proteins10,11.

*P. aeruginosa* is an important Gram-negative opportunistic pathogen that causes serious infections in cystic fibrosis patients, cancer patients, and other immunocompromised individuals13–14. The bacterium remains one of the leading pathogens found in most medical centers, partly because of its ability to develop resistance to conventional antimicrobials. Bacteriophages are a group of viruses that are able to infect and kill bacteria15–17. They have long been proposed as promising alternatives to conventional antibiotics for treating bacterial infections. Given increasing levels of drug resistance in a variety of pathogens and the shortage of new antimicrobials, phage research, particularly phage therapy, has recently gained intense research interest18–20. Thus, efforts to exploit the application of phage therapy in the treatment of bacterial infections have steadily grown. Phage resistance, an essential survival strategy of bacteria, is an important aspect of bacterial-phage interactions and poses serious obstacles in the application of phage therapy21. Therefore, a better understanding of the mechanism of phage-resistance is critical in helping design successful phage-therapy strategies to treat/prevent bacterial diseases, including *P. aeruginosa*-related infections22.

We have recently isolated and sequenced three *P. aeruginosa* phages to explore their biological properties and interaction with host bacteria1,2,24. In this study, using one of the lytic phages (PaP1) and a clinical isolate of *P. aeruginosa* strain (PA123), we identified a novel phage resistance mechanism by chromosomal DNA deletion.
Results

PaP1 infection of *P. aeruginosa* resulted in red-pigment producing, phage-resistant mutants (Red mutants). To investigate phage resistance mechanism in *P. aeruginosa*, wild-type strain PA1 was infected with phage PaP1 (MOI = 1000) and screened for phage-resistant mutants, which could be consistently recovered in high frequency (3.02 × 10⁻²). Interestingly, a high percentage (31% ± 13.2%) of the mutants produced red pigment, and two coupled phenotypes can be stably maintained, indicating genetic mutations. One Red mutant, PA1r, was randomly selected for further analysis, and its PaP1 resistance, as well as red-pigment producing phenotypes, was confirmed by dot assay on an agar plate (Fig. 1B).

Comparative genomic analysis revealed a 219.6 kb genomic fragment deletion in PA1r. To identify the mutation(s) in PA1r that might confer PaP1 resistance, as well as red-pigment producing phenotypes, the genomes of both PA1 and PA1r were sequenced (GenBank: PA1: CP004054, PA1r: CP004055) and subjected to comparative genomic analysis. A 219,572 bp genomic fragment deletion (6,217,866 to 6,437,440) in PA1r compared with PA1 (Fig. 2A) was observed. Sequences flanking the end points are shown in Fig. 2A.

The deletion in PA1r was confirmed by pulsed-field gel electrophoresis (PFGE) (Fig. 2B) and polymerase chain reaction (PCR) (Fig. 2C, Primer sets in Materials and Methods). The precise deletion site in PA1r was verified by PCR (Fig. 2D, Primer sets 6U/6D). Results showed that the deleted fragment in PA1r was flanked by gene *cupA3* and *PALES_33891* compared with another type of strain LESB58[15].

Loss of *hmgA* gene as a result of genomic fragment deletion is responsible for red pigment production in PA1r. Bioinformatic analysis revealed that the deleted 219.6 kb genomic fragment...
contains 192 genes (Fig. S1), including hmgA, a gene encoding the enzyme homogentisate-1,2-dioxygenase that converts homogentisic acid into 4-maleylacetoacetate. Inactivation of this gene results in the accumulation of homogentisic acid, which gives the cell a red pigmentation phenotype26,27.

An hmgA insertional deletion mutant (PA1ΔhmgA) was generated. PA1ΔhmgA produced red pigment but still retained sensitivity to phage PaP1 (Fig. 3), and complementation of hmgA gene in PA1r background did not restore the phage-resistant phenotype (Fig. 3). These results suggest that hmgA gene deletion does not confer PaP1 phage resistance.

Transposon mutagenesis revealed the involvement of PA1 lipopolysaccharides (LPS) in phage PaP1 infection. A random transposon mutant library of P. aeruginosa PA1 was infected with phage PaP1 to identify genes in P. aeruginosa that are essential for phage PaP1 infection. Two phage-resistant mutants (B10 and T2) (Table 1) were isolated and transposon insertion sites were determined by a two-step semi-degenerate PCR28. The mutation in B10 and T2 was mapped to genes PA0938 (wzz2) and PA5001, respectively, both of which have been shown to be involved in LPS biosynthesis29. Given that LPS often serve as phage-receptors in a variety of bacterial species30,31, our data suggested that PaP1 could potentially recognize PA1 LPS as receptors and initiate the infection process.

PA1r carries defective LPS and can prevent phage adsorption. Agglutination test32 was performed with wild-type PA1 and PA1r strains to determine whether the resistance of PA1r to PaP1 infection was due to its defect in LPS biosynthesis. Results showed that PA1 remained as single cells, whereas PA1r tended to form cell clumps (Fig. 4A), suggesting that PA1r carries defective LPS.

Adsorption assays also revealed a significant reduction in phage adsorption to PA1r compared with wild-type PA1 strain (Fig. 4B). The result of the binding assay was further confirmed by transmission electron microscope data. After 3 min co-incubation, a significantly higher number of phage PaP1 particles can be seen attached to the surface of PA1 cell than those binding to PA1r (Fig. 4C). Therefore, PA1r is resistant to this phage.

Loss of galU in PA1r is responsible for phage resistance. Agglutination assay and phage adsorption data suggested that a possible defect in LPS biosynthesis might confer PaP1 phage resistance in PA1r. A detailed bioinformatic analysis revealed within the deleted fragment the presence of galU, a gene involved in LPS biosynthesis. This gene encodes UDP-D-glucose pyrophosphorylase which converts Glc-1-phosphate to UDP-D-Glc, which is the predominant sugar in the outer core of oligosaccharides. A galU mutant is devoid of O-antigen and synthesizes a defective LPS core33–34. Thus, a galU insertional deletion mutant (PA1ΔgalU) was constructed. PA1ΔgalU showed a defect in LPS and was resistant to phage PaP1 infection. Moreover, galU was complemented into PA1r resulting in PA1rΔgalU, which was sensitive again toward this phage (Fig. 3). Therefore, PA1r is significantly attenuated in a mouse infection model. Our data suggested that PA1r acquired phage resistance by deleting a large genomic fragment that contains galU gene essential for LPS biosynthesis. Although the defect in LPS confers resistance to phage infection, this defect might reduce the mutant’s ability to infect a host, because LPS is an important virulent factor in Gram-negative bacterial pathogens, including P. aeruginosa. To test this hypothesis, PA1r was used to infect mice intraperitoneally, and their survival was monitored. All eight mice died within 24 h after infection with either PA1 or PA1rΔgalU, whereas all eight mice survived the infection with PA1r or PA1ΔgalU (Fig. 6).

Red mutants are likely to arise spontaneously before phage infection. Fluctuation test was performed to demonstrate whether the genomic fragment deletion is induced after phage infection. As is shown in Figure 7, the number of red colonies on each plate varied drastically, from 2 to 45 colonies, in Group A; while the variation from group B is very small. This implies that deletion mutation arises before phage infection.

Discussion
In this study, a series of phage-resistant P. aeruginosa mutants were isolated, and almost 30% of these mutants produced red pigment. However, by reviewing the phage resistance mechanisms35,36, no gene has been related to pigment production. Comparative genomic analysis revealed a 219.6 kb genomic fragment deletion in resistant mutant PA1r, including hmgA, which has been demonstrated to be associated with red pigment production (Fig. 3). However, the loss of hmgA could not confer phage resistance to PA1r (Fig. 3).

We further demonstrated that the loss of galU, a key gene involved in LPS biosynthesis, which is 19 kb in front of hmgA, confers phage resistance to PA1r by preventing phage adsorption because LPS is the likely receptor of PaP1 (Fig. 3). Adsorption to the receptor is the initial step for phage infection. Several phage receptors in Gram-negative bacteria, such as LPS, capsular polysaccharides, flagella, and outer membrane proteins5, have been identified. Mutation of the receptor is a common strategy for bacteria to avoid phage predation36. We used an agglutination assay to show that PA1r carried defective LPS (Fig. 4A). Given that the core oligosaccharide is abundant with negatively charged groups of 2-keto-3-deoxyoctulosonic acid and phosphate, strains that lack O polysaccharide will expose core oligosaccharide to the cell surface and cover up the negative charge.

Table 1 | Transposon mutants resistant to phage PaP1 infection

| Transposon mutant | Gene mutated | Function encoded | Pathway involved |
|--------------------|--------------|-----------------|-----------------|
| B10                | PA0938 (wzz2)| Involved in O-antigen chain length determination | LPS biosynthesis[29] |
| T2                 | PA5001       | Putative glycosyl/glucosyltransferase | LPS biosynthesis[29] |
charges. Therefore, LPS negative strains could be agglutinated by high salt concentrations.31

One of the major concerns regarding phage therapy is that phage-resistant mutants might result in the failure of phage therapy21. According to our experiment, phage-resistant bacteria are swift to emerge, and the frequency is much higher than those of drug-resistant mutants. However, phage-resistant mutant PA1r has defective LPS, and LPS is a major virulence factor in P. aeruginosa. Thus, LPS minus mutant PA1r is significantly attenuated in the mouse model (Fig. 6). The trade-off between phage resistance and fitness, such as growth rate or virulence, has also been reported in other phage-host experiments36, which suggests an advantage of phage therapy.

From a genetic perspective, the genomic fragment deletion as a response to phage predation and the underlying mechanism of phage resistance have not been reported. Phage resistance is often acquired by mutations1, DNA fragment insertion (CRISPR)37, or genomic rearrangements6. Our study demonstrated that bacteria could defend phage infection by deleting their own genomic fragment containing...
galU gene essential for phage infection. The two main causes of genomic fragment deletion are spontaneous and site-specific. Spontaneous deletion is caused by the dysfunction of DNA replication and repair system. In this case, the deletion site is random and the deletion rate is quite low. For example, Tanji et al. isolated a phage-resistant Escherichia coli strain D198, which revealed a 209.4 kbp spontaneous deletion. However, this deletion was spontaneous and not repeatable. In an evolution experiment with E. coli after 20,000 generations, only seven deletions were found, ranging from 1 bp to 23,293 bp. In Salmonella enterica, the DNA loss rate was estimated to be 0.05 bp per chromosome per generation. Sanna et al. estimated the apparent deletion rate in S. enterica with chromosomal variation ranging from $0.5 \times 10^{-7}$ to $2.2 \times 10^{-8}$/cell/generation. Another experiment with P. aeruginosa also revealed random fragment deletion, but the deletion sites were unspecific and the frequency was quite low.

By contrast, site-specific deletion is mediated by prophage or other mobile genetic elements. These large DNA fragments are often genomic islands acquired by horizontal gene transfer and could be excised and integrated at a specific site mediated by integrase and other enzymes. This kind of deletion may usually occur at a high frequency. For example, a 89 kbp pathogenicity island in Streptococcus suis, which is flanked by a 15 bp direct repeat, could be excised from the genome with a certain frequency ($\sim 3.2 \times 10^4$).

In our experiments, the deletion events showed three clear novel features: high frequency ($10^{-6}$), large genomic fragment deletions (200 kb to 300 kb), and no site-specificity. Thus, this deletion is neither site-specific nor spontaneous. Interestingly, smaller deletions are certainly favored in bacteria because the cost would be smaller, although the deletions in our experiment ranged from 200 kb to 300 kb. However, by reviewing the genes within the deleted region, none of the genes, including toxin genes, are obviously deleterious (Fig. S1). Therefore, this might be a novel deletion mechanism, and it is currently investigated in our laboratory.

Another interesting issue is whether the observed deletion was induced by phage infection or occurred before phage predation. The fluctuation test is a classical experiment to prove that phage resistant mutants arise before phage infection. If genomic fragment deletion was caused by an induced activation in bacteria, then each plate should contain roughly the same number of red colonies, because the deletion frequency should be consistent in the bacteria population. However, we found that the number of red colonies on each plate varied drastically, from 2 to 45 colonies in Group A; while the variation from group B is very small (Figur 7). This implies that deletion mutation arises before phage infection. What’s more, the replication time of bacterium PA1 is 40 to 60 min at log phase (Figure S2), and the infection cycle of phage pap1 is about 40 min. Thus, within 40 min, the bacterium is unlikely to be able to delete its genomic fragment and losses LPS, because phage already killed bacteria before they replicate. Thus, it seems that the deletion mutants might have already existed in the bacterial population before phage infection.
The more intriguing question is why bacteria delete such a big fragment of their genome in the absence of phage. It is likely that phage resistant mutants were selected before phage treatment due to as-yet-unknown fitness benefits. In an effort to determine whether the selection could be due to mutants’ growth advantage in culture medium, we compared the growth rate of PA1 and the deletion mutants before phage infection still needs further investigation in this system. In summary, we reported a novel phage resistance mechanism in which P. aeruginosa evades phage infection by genomic fragment deletion, a finding that could provide valuable insight into phage therapy.

### Methods

#### Bacteria and bacteriophage growth conditions

Phages and hosts used in this study are listed in Table 2. *P. aeruginosa* and lytic phage PaP1 were stocked in our laboratory. *P. aeruginosa* strains were grown on Luria-Bertani (LB) agar plates or in LB broth with aeration at 37 °C. When required, ampicillin, gentamicin, or tetracycline were used. Their concentrations in different media are described below.

#### Selection for phage-resistant mutants

*P. aeruginosa* PA1 was grown at 37 °C in LB broth with aeration. When the bacterial culture reached OD600 of 0.2, 10 μl of culture (approximately 1 × 10^8 cfu) was mixed with 100 μl and 10^6 psu/ml phage PaP1. The phage-infected culture was incubated at 4 °C for 5 min and spread to one LB agar plate. Plates were then grown at 37 °C for 24 h. Phage sensitivity of the isolated colonies were tested as previously described^11^. Briefly, 3 ml of 0.8% soft agar was mixed with 50 μl overnight culture of bacteria and was layered onto bottom agar. Finally, 10 μl of phages were deposited on the agar and incubated overnight at 37 °C.

#### DNA extraction

Genomic DNA from *P. aeruginosa* wild-type strain PA1 and phage-resistant mutant PA1r were extracted using UNlQ-10 Column Bacterial Genomic DNA Isolation Kit (SK1202; Sangong Biotech, Shanghai) according to the manufacturer’s instructions. The quality and quantity of extracted DNA were measured using spectrophotometry. Extracted DNA with a concentration of up to 500 ng/μl was used for the whole genome sequencing and PCR verification.

#### High-throughput genome sequencing

The complete genomes of the wild-type strain PA1 and the PaP1-resistant mutant PA1r were sequenced using Solexa to a depth of 200× (median coverage 161×), according to the manufacturer’s protocols. The reference genome of *P. aeruginosa* strain LESB58 (GenBank accession: NC_011770) was downloaded from NCBI and used for mapping the short sequencing reads produced by the Illumina Genome Analyzer. The sequencing reads of each isolate were mapped separately to their corresponding reference genome.

#### Verification of genomic fragment deletion by PCR

Primers 1U/1D, 2U/2D, 3U/3D, 4U/4D, and 5U/5D (Table 3) were designed to amplify five different regions of each isolate were mapped separately to their corresponding reference genome.

### Table 2 | Bacterial strains, plasmids, and phage used in this study

| Bacterial strain or plasmid | Relevant feature(s)^* | Reference or source |
|----------------------------|------------------------|---------------------|
| **P. aeruginosa Strains**   |                        |                     |
| PA1                        | Wide type strain for phage PaP1 | [23]               |
| PA1r                       | Phage resistant mutant  | This study          |
| PA1R-37                    | Phage resistant mutant  | This study          |
| PA1R-57                    | Phage resistant mutant  | This study          |
| PA1R-61                    | Phage resistant mutant  | This study          |
| PA1r-hmgA                  | Insertional deletion of hmgA | This study          |
| PA1r-galU                  | Insertional deletion of galU | This study          |
| PA1r: hmgA                 | Complementation of hmgA | This study          |
| PA1r: galU                 | Complementation of galU | This study          |
| E. coli strains            |                        |                     |
| S17-1 (p pir)              | Conjugative donor strain | [51]               |
| SM10Op8                    | Conjugative strain      | [28]               |
| **Bacteriophage**          |                        |                     |
| PaP1                       | Lytic phage            | [23]               |
| **Plasmid**                |                        |                     |
| pUCP24                     | 4.036-kb pUC18-based broad-host-range vector, Gm' | [52]               |
| pEX18Tc                    | Gene replacement vector, Tc' | [51]             |
| pCM639                     | ISpolA: hoh-Tc delivery plasmid | [28]       |
| pucci-galU                 | Broad-host-range vector containing galU, Gm' | This study         |
| pucci-hmgA                 | Broad-host-range vector containing hmgA, Gm' | This study         |
| pEX-galU                   | galU deletion vector, Tc' | This study          |
| pEX-hmgA                   | hmgA deletion vector, Tc' | This study          |

Abbreviations: Gm', gentamicin resistance. Tc', tetracycline resistance.

### Table 3 | Primers for detecting the deletion sites in phage resistant mutants

| Primer name | Sequence |
|-------------|----------|
| 1U          | GCGGAGGGCGGAAGAATGAAAGC |
| 1D          | TTGACACGGCAAGGGGAAAGC |
| 2U          | AGGTTGCACTGATACGGGTTC |
| 2D          | CGTCCTAGTCTTCTGTCGTC |
| 3U          | GCCCTGACAGGCGAAAGGAAAGA |
| 3D          | GCCGAGCCGACGGAAAGACAG |
| 4U          | CGCGTACAGGCTACAGGAAAC |
| 4D          | TICTCCTGCGGCAACGCGCA |
| 5U          | GCCACCGGAAATGTACGACAA |
| 5D          | AGCGGAAACCAAGACAGCACG |
| 6U          | CTGGGATTTGAGACAGGACCTAC |
| 6D          | GGTAGCTGATGAGTAGGGGTTG |
| 7U          | GCAACAGGCGACAGGAGGAGG |
| 7D          | GCCTGAGCCGACGACGACAG |
| 8U          | GCCACAGTCAAATACACTCA |
| 8D          | AC CGGCAACGGGAGTAGT |
| 9U          | ATGATCAAAATGTCTTCGCC |
| 9D          | TCACTGACGCTCCGCCTTCTG |
| 10U         | AATTAGAGCTGTCGCCCT |
| 10D         | GTAGTGCCTAGTGGACG |
| 11U         | ACTATGGAACGACGAAAGC |
| 11D         | CGGTAGGTCGTCGTCGTC |
precise deletion site in PA1r. Primer sets (7U/7D, 8U/8D, 9U/9D, 10U/10D, and 11U/11D; Table 3) were designed to detect different deletion sites in different Red mutants. Their locations are indicated in Figs. 2A and 5A.

**PFGE.** PFGE was performed as previously described with the following modifications. After digestion of the genomic DNA with SpeI, PFGE was performed at 14°C with CHEF MAPPER XA (Bio-Rad) at 200 V with switch times that ranged from 5 s to 52 s for 18 h.

**Transposon mutagenesis.** Transposon mutagenesis was performed as previously described with the following modifications. After mixing *P. aeruginosa* PA1 with *E. coli* strain SM10pir/pCM639 for 2 h, the cells were collected and resuspended in 1 ml of LB. Bacterial cells were then incubated with a 30-fold excess of the phage PaP1 for 20 min at 37°C and plated on LB medium containing tetracycline (65 μg/ml) and chloramphenicol (10 μg/ml) for the inhibition of the donor strain. The insertion of the transposon was identified by a two-stage semi-degenerate PCR and sequencing as described previously.

**Electron microscopy.** Phage PaP1 and bacterial strain PA1 or PA1r were co-incubated for 3 min and placed on copper screen for 10 min before being negatively stained with 2% phosphotungstic acid (pH 4.8) for 1 min. The samples were then incubated for 3 min and placed on copper screen for 10 min before being negatively stained with 2% phosphotungstic acid (pH 4.8) for 1 min. The samples were then scanned under TECNAI 10 electron microscope (Philips, The Netherlands) at 80 kV.

**Fluctuation test.** Log phase bacteria PA1 was diluted to 10^5 cell/ml. Then inoculate 0.5 ml into each of the 20 tubes (Group A), and inoculate 5 ml into one tube (Group B). When the culture reach OD600 = 0.2, 20 μl of cell culture from each tube in Group A was plated onto agar containing the PaP1 phage. For group B, all the 20 replicates were performed used the bacteria from the same tube. Then the red colonies were calculated after 24 h incubation.

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