Characterization of *Pseudomonas aeruginosa* Phage C11 and Identification of Host Genes Required for Virion Maturation

Xiaoli Cui, Jiajia You, Li Sun, Xiaojing Yang, Tian Zhang, Kechong Huang, Xuewei Pan, Fenjiao Zhang, Yang He & Hongjiang Yang

The underlying mechanisms of phage-host interactions largely remained to be elucidated. In this work, *Pseudomonas aeruginosa* phage C11 was first characterized as a *Myoviridae* virus having a linear dsDNA molecule of 94109 bp with 1173 bp identical terminal direct repeats (TDR). Then the mutants resistant to phage C11 were screened in a Tn5G transposon mutant library of *P. aeruginosa* PAK, including two mutants with decreased adsorption rates (DAR) and five mutants with wild-type adsorption rates (WAR). When the WAR mutants were incubated with phage C11, their growth rates were significantly inhibited; the replication of the phage genomic DNA was detected in all the WAR mutants with the real-time quantitative PCR analysis; and the synthesized phage genomic DNA was processed into monomers for packaging evidenced by the southern blot analysis. Moreover, with strain PAK as indicator, small quantities of phage C11 were synthesized in the WAR mutants. Taken together, these data suggested the identified genes of the WAR mutants are necessary for efficient synthesis of the infectious phage particles. Finally, the WAR mutants were detected sensitive to two other *Pseudomonas* phages closely related with C11, further implying the evolved diversity and complexity of the phage-host interactions in both sides.

Phage therapy shows great promises in combating bacterial infections1-2. Candidate phages used for treatments are usually selected mainly based on their host ranges. Phages with broader host ranges will likely target more bacterial strains and may have more potential applications in practice. In addition to killing spectrums of phages, antibacterial efficacy of phages is another key aspect of candidate phages for consideration of phage therapy, which is involved a variety of genes from both sides in host-phage interactions3-4. A number of mechanisms have been found contributing the defense of phage attacks in many bacteria5-6. All these involved pathways are employed by diverse bacteria strains in the active strategies against phage infections. The host genes necessary for phage proliferations largely remain to be identified and elucidated.

The systematic investigations of phage-host interactions between *Escherichia coli* and the relevant phages have been performed recently. Two bacterial libraries, the Keio collection and the ASKA library, have been used for the genome-wide searches of host genes involved in the phage T7 infection, including some genes for their ability to inhibit growth of T7 phage and the other genes required for the virus infection7. The Keio collection was also used to identify bacterial genes involved in the λ phage infection process. Totally 57 host genes were identified, the majority of them had not been found associated with phage infections previously8. Phage receptor related genes were also screened in *E. coli* phage mEp213 infection by employing a novel strategy to select bacterial cell-envelope mutants resistant to phage infection9. More recently, it’s found that the genome injection of *E. coli* phage HK97 required the glucose transporter PtsG and the periplasmic chaperone FkpA encoded by the host genes10.

Similar studies on host-phage interactions have also been carried out in various systems, including *Bacillus subtilis* and phage SPP111, *Yersinia pestis* and phage L–413C12, *Vibrio cholerae* and phageVP313, *V. cholerae* biotype El Tor and phage VP414, and *Salmonella enterica* serovar Typhi and a number of diverse phages15-17. All identified
host genes required for phage infections approximately fall into two groups, one group of the genes related to the receptors syntheses, involving in phage recognitions and adsorptions; the other group of the genes involved in various pathways, possibly functioning in the stages of phage infection other than the adsorption.

_Pseudomonas aeruginosa_ is an opportunistic pathogen causing a number of diseases in human beings and also one of the most common bacteria causing nosocomial infections. _P. aeruginosa_ possesses a relatively large genome harboring multiple drug resistance determinants, leading to the ever increasing prevalence of multi-drug resistances in clinical isolates along with the imprudent and excessive use of antibiotics. It’s urgent to identify antibiotic alternatives with efficacious antibacterial activities. Phages are able to specifically kill bacteria hosts with high efficiency and expected to be used in bactericidal treatments as the biological agents. To date, a few clinical trials have been carried out using phages against _P. aeruginosa_ infections with the encouraging results, such as leg ulcers, burns, and ear infection. Phage treatment has also been conducted to control _P. aeruginosa_ infection in cystic fibrosis patients in a few cases. New techniques have been applied in the exploration of host-phage interactions in a few systems, including _P. aeruginosa_ and phage LUZ19, _P. aeruginosa_ and phage PaP30, and _P. aeruginosa_ and phage PAK P33. The data reveal the global changes in host cells when infecting with the virulent phages. A number of phage genes are found playing crucial roles during the infections. On the other hand, host genes necessary for phage infections have also been investigated in some _Pseudomonas_ strains with classical genetic techniques. The majority of the identified genes are related to the synthesis of phage receptors, such as type IV pili and LPS. In screening phage JG004 resistant mutants, the gene _speD_ encoding S-adenosylmethionine decarboxylase proenzyme for polyamine biosynthesis was identified, and the enzyme is possibly involved in the phage genome packaging process by affecting the charge density of the genomic DNA molecules.

Phage C11 is a virulent virus isolated previously using the clinical strain _P. aeruginosa TJC422_ as indicator. In this work, the characteristics of phage C11 were evaluated. The genome of phage C11 was sequenced, curated, and annotated. In the investigation of the host-phage interaction, the mutants resistant to phage C11 were screen in a Tn5G transposon insertional library of strain PAK and the interrupted host genes were identified. The mechanisms underlying the infection process was further explored by characterization of the isolated phage-resistant mutants.

**Results**

**Biological characteristics of phage C11.** Transmission electron micrograph (TEM) was used to investigate morphology of phage C11. The images showed that phage C11 had an icosahedral head of 65 nm in diameter and a long contractile tail with a length of 122 nm, indicating that phage C11 can be tentatively classified into the family _Myoviridae_ (Fig. 1A). In the SDS-PAGE analysis of phage particles, 4 major bands and 5 minor bands were observed on the gel, and the protein bands likely represented the major structural proteins of phage C11 (Fig. 1B). Furthermore, the infectivity of phage C11 was characterized by one-step growth experiment at a multiplicity of infection (MOI) of 0.001. In the infection process of strain PAK, phage C11 had a latent period of about 18 min and a burst size of approximately 11 PFU/infection centre (Fig. 1C).

**Termini identification of the phage C11 genome.** The assembled phage genome was 93010 bp in length and it was subsequently confirmed as a linear dsDNA molecule by digestion analysis with the enzyme _PvuI_ (Fig. 2A,B). To further verify the phage genomic structure, the high-frequency sequence (HFS) sites possibly representing the phage genome termini were revealed by analyzing the sequencing depth. One HFS between 82259 bp through 83429 bp of the assembled genome was detected and hypothesized as the genome termini sites. In combination with the restriction mapping analysis, the 6.5 kb _PvuI_ fragment and 2.6 kb _AflIII_ fragment (larger...
than the simulated 1.5 kb fragment) possibly containing the phage termini were purified from gel, respectively. After amplification of the purified fragments, the 1.7 kb and 0.79 kb PCR products were sequenced (Fig. 2A–D).

An identical terminal direct repeat sequence of 1173 bp was found in both the two fragments and the genome size of phage C11 was curated to 94109 bp.

Genome annotation. A total of 172 ORFs encoding protein were predicted of the phage C11 genome, including 30 proteins with known putative functions and 142 hypothetical proteins (Table 1). The overall genome structure showed that genes with similar functions were arranged in clusters, which were divided into five functional modules: the module of nucleotide metabolism related genes; the module of the head and tail of phage and cell lysis related genes; the module of DNA replication, transcription, recombination, and modification related genes; and the remaining two modules having a number of genes localized close to the termini of the genome with unknown functions (Fig. 3). Between the modules of nucleotide metabolism related genes and cell lysis genes, 12 tRNA genes were predicted and closely colocalized within one cluster (Fig. 3). Phage C11 genome was highly homologous to phage JG004 (NC_019450), phage PaP1 (NC_019913), phage vB_PaeM_C2-10_Ab1 (NC_019918), and phage PAK_P1 (NC_015294) at the nucleotide level of 95.32%, 92.41%, 92.11%, and 93.03%, respectively, and with very similar genome structures. However, genes ORF158, ORF159, and ORF165 were unique present in the phage C11 genome encoding the proteins with unknown functions.

Isolation and characterization of phage resistant mutants. By screening the Tn5G transposon insertional library, seven mutants resistant to phage C11 were acquired from the collection of nearly 20,000 mutants. In the spotting assay, strain PAK displayed transparent zones within 4 h incubation; whilst no clear zones were observed in the lawns formed by the mutants. Additionally, no plaques were formed in the double-layer plating assay using the mutants as indicators. The data implied that the isolated mutants showed resistance to phage C11 infections. The adsorption rate of the mutants was determined, two phage-resistant mutants displaying decreased adsorption rates (DAR) of 55.3% and 42.8%, respectively, significantly lower than that of the parent strain PAK (all P values less than 0.01); while the remaining five phage-resistant mutants displaying wild-type adsorption rates (WAR) at the range from 93.3% to 95.4%, no significant difference from the adsorption rate 92.7% of PAK (all P values higher than 0.05) (Fig. 4A).

Identification of the disrupted genes. The Tn5G transposon insertion sites were determined by the inverse PCR method. The DAR mutants RC11-7 and RC11-20 had the decreased adsorption rates, suggesting the disrupted genes might be related with the phage receptor synthesis. The prolonged measurement of the adsorption rate further confirmed that both RC11-7 and RC11-20 had their adsorption ability impaired (Fig. S1). RC11-7 had the transposon inserted in gene PAK_02041 identical to gene wbpR of P. aeruginosa LESB58, which encodes a glycosyltransferase involved in LPS synthesis56. RC11-20 had the transposon inserted in gene PAK_05691 identical to gene PA5181 of P. aeruginosa PAO1, which encodes a probable molybdopterin oxidoreductase and it's the second gene of the operon PA5180-PA5181 (www.pseudomonas.com).
The five WAR mutants all had the wild-type adsorption rates similar to strain PAK, indicating the disrupted genes were not related to the synthesis of the phage C11 receptor. RC11-2 had the transposon inserted in the intergenic region between gene \( PAK_03116 \) and gene \( PAK_03117 \) of PAK. The identical sequence is found and annotated as gene \( BN889_05221 \) of \( P. aeruginosa \) PA38182, which encodes a hypothetical peptide of 75 aa without any known function. RC11-5 had the transposon inserted in the sequence identical to the intergenic region before gene \( PA3808 \) of \( P. aeruginosa \) PAO1, which is the last gene in the operon \( iscR-iscS-iscU-iscA-hscB-hscA-fdx2-PA3808 \). The proteins encoded by the operon are mainly responsible for the iron-sulfur cluster (ISC) assembly. RC11-21 had the transposon inserted in gene \( PAK_00453 \) identical to gene \( PA0243 \) of \( P. aeruginosa \) PAO1, which encodes a TetR bacterial regulatory protein with helix-turn-helix (HTH) signature (PF00440). RC11-22 had the transposon inserted in gene \( PAK_04254 \) identical to gene \( PA1115 \) of \( P. aeruginosa \) PAO1, which encodes a probable sulfatase (PF00884) catalyzing the hydrolysis of sulfate esters. RC11-10 had the transposon inserted in gene \( PAK_03341 \) identical to gene \( PA1993 \) of \( P. aeruginosa \) PAO1, which encodes a probable major facilitator superfamily (MFS) transporter (www.pseudomonas.com).

### Table 1. Phage genes with putative functions.

| Category                                      | ORF         | Function                                              |
|-----------------------------------------------|-------------|-------------------------------------------------------|
| Nucleotide metabolism                         | ORF020      | putative nicotinamide phosphoribosyl transferase      |
|                                               | ORF022      | phosphoribosyl pyrophosphate synthetase               |
|                                               | ORF023      | putative ATPase                                       |
|                                               | ORF024      | putative RNA ligase                                   |
|                                               | ORF028      | putative phosphoesterase                              |
|                                               | ORF030      | putative phosphohydrolase                             |
|                                               | ORF033      | DNA ligase                                            |
|                                               | ORF034      | putative dCMP deaminase                               |
|                                               | ORF044      | putative protease subunit                              |
| Head and tail assembly                        | ORF049      | putative large terminase subunit                      |
|                                               | ORF050      | structural protein                                    |
|                                               | ORF051      | N6 adenine specific DNA methyltransferase             |
|                                               | ORF054      | major capsid protein                                  |
|                                               | ORF056      | putative RNA polymerase                               |
|                                               | ORF059      | putative structural protein                           |
|                                               | ORF065      | putative tape measure protein                         |
|                                               | ORF069      | putative baseplate protein                            |
|                                               | ORF071      | putative base plate related protein                   |
|                                               | ORF073      | putative tail fiber protein                           |
|                                               | ORF075      | putative tail fiber protein                           |
|                                               | ORF086      | putative RNA ligase                                   |
|                                               | ORF101      | DNA primase/helicase                                  |
|                                               | ORF102      | DNA polymerase                                        |
|                                               | ORF109      | putative exodeoxyribonuclease                         |
|                                               | ORF123      | thymidylate synthase                                  |
| DNA replication, transcription, recombination  | ORF125      | ribonucleoside diphosphate reductase beta subunit      |
| and modification                               | ORF126      | ribonucleoside diphosphate reductase alpha subunit     |
| Cell lysis                                     | ORF031      | putative cell wall hydrolase                          |
|                                               | ORF076      | endolysin                                             |

The five WAR mutants all had the wild-type adsorption rates similar to strain PAK, indicating the disrupted genes were not related to the synthesis of the phage C11 receptor. RC11-2 had the transposon inserted in the intergenic region between gene \( PAK_03116 \) and gene \( PAK_03117 \) of PAK. The identical sequence is found and annotated as gene \( BN889_05221 \) of \( P. aeruginosa \) PA38182, which encodes a hypothetical peptide of 75 aa without any known function. RC11-5 had the transposon inserted in the sequence identical to the intergenic region before gene \( PA3808 \) of \( P. aeruginosa \) PAO1, which is the last gene in the operon \( iscR-iscS-iscU-iscA-hscB-hscA-fdx2-PA3808 \). The proteins encoded by the operon are mainly responsible for the iron-sulfur cluster (ISC) assembly. RC11-21 had the transposon inserted in gene \( PAK_00453 \) identical to gene \( PA0243 \) of \( P. aeruginosa \) PAO1, which encodes a TetR bacterial regulatory protein with helix-turn-helix (HTH) signature (PF00440). RC11-22 had the transposon inserted in gene \( PAK_04254 \) identical to gene \( PA1115 \) of \( P. aeruginosa \) PAO1, which encodes a probable sulfatase (PF00884) catalyzing the hydrolysis of sulfate esters. RC11-10 had the transposon inserted in gene \( PAK_03341 \) identical to gene \( PA1993 \) of \( P. aeruginosa \) PAO1, which encodes a probable major facilitator superfamily (MFS) transporter (www.pseudomonas.com).

**Complementation of the phage resistant mutants.** To verify if the interrupted genes were responsible the phage-resistant phenotypes, the target genes were cloned into the vector pUCP18 with their natural promoters or driven by the Plac promoter carried by the plasmid (Table 2) and the recombinant plasmids were transformed into the mutants. The resulting transformants were analyzed for their sensitivity to phage C11. The analyses showed that the sensitivity to phage C11 was restored in all seven phage-resistant mutants by the trans-complementation of the corresponding target genes, demonstrating the disrupted genes were responsible for the phage-resistant phenotype of the mutants (Fig. 4B).

**Phage C11 affecting the growth dynamics of the WAR mutants.** The fact that the WAR mutants had the wild-type phage adsorption rates indicated that the impaired genes were not involved in the process of phage adsorption. These genes were possibly associated with the stages of the phage genome injection, replication, packaging, or progeny release. Based on this hypothesis, the WAR mutant cells were mixed with phage C11 at different MOIs and the growth rates were measured. As the MOIs increased from 1 to 100, the WAR mutants grew slower as the more phage particles added (Fig. 5). The data showed that the phage-resistant WAR mutants exhibited...
Figure 3. Analysis of the phage C11 genome. The outmost two rings showed CDS features in blue boxes on both the plus and minus strands. CDS with no annotations represented hypothetical proteins. The clustered transfer RNA genes were abbreviated as Gln (glutamine), Arg (arginine), Leu (leucine), Ile (isoleucine), Asp (aspartic acid), Cys (cysteine), Asn (asparagine), Pro (proline), Gly (glycine), Phe (phenylalanine), Glu (glutamate), and Thr (threonine). The black ring showed the GC content of the phage C11 genome. The inmost ring showed the value of GC skew, green color standing for positive and purple for negative.

Figure 4. Characterization of the phage resistant mutants. (A) Phage adsorption rate of the mutants and the parent strain PAK. The experiment was repeated three times. (B) Complementation experiment. The upper panel showed the mutants were resistant to phage C11. The lower panel showed the sensitivity restoration to phage C11 in the corresponding transformants. White triangles pointed at the clear zones caused by spotting the 100-fold diluted phage C11 lysate (10⁸ pfu/ml) on the double-layer plates.
C11 genomic DNA may undergo normally in all the WAR mutants. from the *Apa*I digested monomeric DNA (Fig. 7). All the data showed that the packaging process of the phage genomic DNA was synthesized in the WAR mutants without forming clear zones in the spotting assay or plaques in the double-layer plating analysis. The phage infection process might be blocked at the stages after genome replication. Southern blot analysis was performed to explore the packaging process of the synthesized phage genomic DNA. The data showed that the phage C11 genomic DNA was replicated intracellularly in the WAR mutants and strain PAK. Compared with the parent strain PAK, the five WAR mutants all had the genomic DNA replicated with different efficiencies, ranging from 47.6% to 68.6% (all P values less than 0.01) (Fig. 6). The results confirmed that the phage C11 genome had been successfully injected into bacterial cytoplasm and undergone DNA synthesis in the WAR mutants.

Quantitative assessment of intracellular phage genomic DNA. To further probe if the phage genomic DNA was replicated in the WAR mutants, the cultures containing the WAR mutants and phage C11 were sampling at 0 min and 30 min for total DNA extraction, respectively. The same amount of DNA was used in the real-time quantitative PCR (RT-qPCR) analysis. At 0 min, all samples collected had the cycle threshold (Ct) value ranging from 17.68 to 21.17, indicating similar amounts of the phage particles were attached to the cellular surface of all strains. All samples collected at 30 min showed the Ct value ranging from 17.68 to 21.17, indicating the WAR C11 genomic DNA was replicated intracellularly in the WAR mutants and strain PAK. Compared with the parent strain PAK, the five WAR mutants all had the genomic DNA replicated with different efficiencies, ranging from 47.6% to 68.6% (all P values less than 0.01) (Fig. 6). The results confirmed that the phage C11 genome had been successfully injected into bacterial cytoplasm and undergone DNA synthesis in the WAR mutants.

Genome packaging analysis of the replicated DNA. The phage genomic DNA was synthesized in the WAR mutants without forming clear zones in the spotting assay or plaques in the double-layer plating analysis. The phage infection process might be blocked at the stages after genome replication. Southern blot analysis was performed to explore the packaging process of the synthesized phage genomic DNA. The data showed that the pure phage genomic DNA digested with *Xho*I, *Apa*I, and *EcoRV*, respectively, displayed the positive bands from the mixtures containing bacteria and phage C11, two positive bands were detected with the designed DNA probe, including the 10.0 kb fragment and the 6.5 kb fragment (Fig. 7A). The data was consistent with the predictions as shown in the scheme (Fig. 7B). The 10.0 kb fragment was possibly from the phage C11 genomic DNA was replicated normally in all the WAR mutants.

Infection efficiency analysis in the WAR mutants. No transparent zone was observed in the lawn of the WAR mutants when infected with phage C11 in the spotting assay (Fig. 4B). The result was further confirmed by the fact that no plaques were detected in the standard double-layer plating method using the WAR mutants as indicators. However, turbid and small plaques were observed when plating the WAR mutant–phage complexes using strain PAK as the indicator, similar to the mixed-indicator technique. The modified one-step growth experiment was performed to characterize the infectivity of phage C11 in the WAR mutants using wild type strain PAK as indicator instead of the WAR mutants. The WAR mutants RC11-2, RC11-5, RC11-10, RC11-20, RC11-21, and RC11-22 all could produce and release low quantities of phage C11, with the lower infection efficiency of 3.4%, 38.5%, 41.5%, 33.3%, and 29.0%, respectively (all P values less than 0.01) (Fig. 8A). The phage production ability was also estimated in the WAR mutants. The phage titres at 35 min were 13.8, 6.7, 7.3, 9.9, 17.7, and 14.2

### Table 2. Strains, phage, and plasmids used in this study.

| Strain, phage, and plasmid | Description | Reference |
|---------------------------|-------------|-----------|
| *P. aeruginosa* | | |
| PAK | Laboratory strain | 73 |
| RC11-2 | *BN889_05221::Tn5G*, phage C11 resistant mutant of PAK, Gm’ | This study |
| RC11-5 | *PA5808::Tn5G*, phage C11 resistant mutant of PAK, Gm’ | This study |
| RC11-7 | *wbpR::Tn5G*, phage C11 resistant mutant of PAK, Gm’ | This study |
| RC11-10 | *PA1993::Tn5G*, phage C11 resistant mutant of PAK, Gm’ | This study |
| RC11-20 | *PA5181::Tn5G*, phage C11 resistant mutant of PAK, Gm’ | This study |
| RC11-21 | *PA0243::Tn5G*, phage C11 resistant mutant of PAK, Gm’ | This study |
| RC11-22 | *PA1115::Tn5G*, phage C11 resistant mutant of PAK, Gm’ | This study |

| Phage | | |
|-------|-------------|-----------|
| C11 | *P. aeruginosa* lytic phage | 33 |

| Plasmid | | |
|---------|-------------|-----------|
| pUCP18 | Broad host range shuttle vector, Ap’ | 74 |
| pRR2013Tn5G | used for transposon mutagenesis, Gm’ | 71 |
| pXL1502 | *BN889_05221* gene driven by *Pprom*, promoter cloned in pUCP18, Ap’ | This study |
| pXL1503 | *PA5808* gene driven by *Pprom*, promoter cloned in pUCP18, Ap’ | This study |
| pXL1504 | *wbpR* gene with promoter region cloned in pUCP18, Ap’ | This study |
| pXL1505 | *PA1993* gene with promoter region cloned in pUCP18, Ap’ | This study |
| pXL1506 | *PA5181* gene driven by *Pprom*, promoter cloned in pUCP18, Ap’ | This study |
| pXL1507 | *PA0243* gene driven by *Pprom*, promoter cloned in pUCP18, Ap’ | This study |
| pXL1508 | *PA1115* gene with promoter region cloned in pUCP18, Ap’ | This study |
folds to that at 5 min in RC11-2, RC11-5, RC11-10, RC11-21, RC11-22, and PAK, respectively, indicating phage progenies were obviously synthesized in the mutants (all P values less than 0.05) (Fig. 8B).

Sensitivity analysis of the WAR mutants to phage K5 and K8. *P. aeruginosa* phage K5 and K8 were highly homologous to phage C11 at the genomic level and with respect of most individual genes with putative know functions, hence these viruses may share similar infection processes. However, with the spotting assay, the WAR mutants were found sensitive to the both phage K5 and K8, implying that the identified host genes were not required for the infection by phage K5 and K8. The data further showed the diversity of phage-host interactions.

Discussion

*P. aeruginosa* phage C11 is highly similar to the genome of *P. aeruginosa* phage PaP1 and may be considered as a new member of the genus PaP1-like phages or PAK_P1-like phages. Though the phages C11, JG004, PaP1, vB_PaeM_C2-10_Ab1, and PAK_P1 are genetically closely related to each other, they are the different phage isolates recovered across the world with their own evolutionary paths. Phage C11 has 3 unique genes ORF158, ORF159, and ORF165 in its genome. At the amino acid level, the hypothetical protein ORF158 has an identity of 41.12% with the pantothenate kinase encoded in *Prevotella falsenii*; ORF159 has an identity of 38.32% with the hypothetical protein encoded in *Pseudomonas veronii*; and ORF165 has an identity of 44.15% with the hypothetical protein PA13_1003640 encoding in *Pseudomonas aeruginosa* HB13. These genes may have their own bacterial origins and possibly be obtained by phage C11 via the process of horizontal gene transfer (HGT). The complete genome sequence of phage C11 is shown to be a liner DNA molecule with the 1173 bp identical terminal direct

Figure 5. The inhibitory effect of phage C11 on the growth rates of the WAR mutants and the parent strain PAK. MOI: the multiplicity of infection.

Figure 6. The quantitative analysis of the replication efficiency of the phage genomic DNA in strain PAK and the WAR mutants. The replication efficiency of the phage C11 genomic DNA in the WAR mutants was estimated by comparing with strain PAK after 30 min infection by phage C11.
repeats, similar to a variety of *P. aeruginosa* phages which have highly conserved direct repeat sequences with length ranging from 184 bp to 1238 bp.

A number of *Pseudomonas aeruginosa* phages recognize LPS as receptors for adsorption during infection, such as phage JG02441, JG00432, PaP136, and KT28 and KTN631. In our study, gene *wbpR* was found inactivated in the DAR mutant RC11-7. WbpR is a glycosyltransferase responsible for transferring the fourth residue L-Rhamnose of one O antigen repeating unit in the biosynthesis of OSA LPS, indicating that LPS was the receptor for phage C11 binding. PA5181 is a putative molybdopterin oxidoreductase sharing a similarity of 76.11% with protein

**Figure 7. Analysis of the packaging process of the replicated genomic DNA by Southern blot.** (A) total DNA was extracted from the samples (1–9), digested with the restriction enzyme *ApaI*, and detected by the synthesized DNA probe. The 10 kb *ApaI* fragment was predicted from the digested concatemeric DNA. The 6.5 kb *ApaI* fragment was predicted from the digested single viral genome (monomeric DNA). Marker: 15 kb DNA marker. (B) the schematic representation of the predicted phage genomic structures and the corresponding *ApaI* fragments detected in the Southern blot analysis. α: concatemeric DNA. β: monomeric DNA. The solid purple arrows stood for the terminal direct repeats (TDR) of the phage genome. The red rectangles stood for the DNA probe region.

**Figure 8. Infection efficiency assay of phage C11 infection in the WAR mutants.** PAK instead of the WAR mutants was used as the indicator strain at a MOI of 0.00001 in the modified one-step growth experiment. (A) The infection efficiency was evaluated by comparing the phage titers of the WAR mutant cultures with that of the PAK cultures at 35 min. (B) The amount of phage progenies was evaluated by comparing the phage titer at 5 min with that at 35 min.
infectivity, and the ratio affects the determination of the ratio between protein gpG and gpGT of phage λ, which plays an important role in the phage production. For the U34 thiolation process of tRNA Glu, tRNAGln, and tRNALys, the modification of the tRNAs plays an important role in determining the ratio between protein gpG and gpGT of phage λ, and the ratio affects the efficiency of the assembly and release of phage λ progenies. Iron ion can be one of the key components of the functional phage tail like protein complexes. In phage λ, the tail tip complex (TTC) mediates the adsorption and injection of the genomic DNA during the infection process. The TTC protein Gp14 has four conserved Cys residues at its C-terminus for the binding of the ISC proteins in which iron ion is obtained via the protein IscX. In phage T4, seven iron ions were found coordinated by histidine residues in the virion, including the tip of the phage T4 long tail fiber with the crystal structure analysis. In phage P2, the host-binding domain found in the tail-spike protein gpV of P2 phage also reveals a trimeric iron-binding structure with the crystal structure analysis, which suggests the efficient production of phage C11 may require the biological availability of iron ion in the WAR mutant. More experiments are required to elucidate the underlying mechanisms.

There are three types of viral genome packaging systems, including energy-independent packaging system, portal-translocase system, and FtsK/HerA-type packaging system. In our work, the genome of phage C11 was identified to be a linear dsDNA molecule with 1173 bp TDR at its termini. Phages like λ and T7 package a precise genomic unit by cutting at the cos sites, while the other phages like SPP1 and P1 start packaging by cutting at the pac sites and finish packaging based on the size of the packaged DNA via the classical headful packaging mechanism. With the discontinuous headful packaging model, phage T4 can package and transduce two plasmid DNAs with different antibiotic markers via the in vitro packaging experiments. There are packaging limits of genome lengths for phage production. For λ phage, the increasing genomic DNA length leads to the dramatic reduction of phage infectivity.

The head-to-tail concatemeric DNA and monomeric DNA were detected during the replication process with the Southern blot analysis in our work, similar to the genome replication and packaging process found in many tailed dsDNA phages. However, the EcoRI digested genomic DNA showed one unexpected 4.0 kb band, and the DNA samples purified from the phage infection process displayed the consistent results. There were five disrupted genes revealed essential for phage infection but not related to the steps of the infection process via the unidentified pathway(s).

In the WAR mutants, five genes were revealed essential for phage infection but not related to the steps of adsorption and injection. RT-qPCR and Southern blot analyses indicated that phage C11 had its genome replicated and packaged normally in the WAR mutants without detecting of phage progenies by using the standard spotting assay and the double-layer plating method. However, the modified one-step growth experiment method using PAK as indicator showed the WAR mutants can produce small quantities of phage C11, consistent with the data from the RT-qPCR and Southern blot analyses.

The functions of all the five disrupted genes weren’t investigated previously. BN889_05221 of P. aeruginosa PA38182 is a small hypothetical protein with unknown function. PA1115 of P. aeruginosa PAO1 is a hypothetical membrane protein probably with sulfuric ester hydrolase activity. PA1993 is a probable major facilitator superfamily (MFS) transporter sharing a homology of 50.2% with the L-arabinose efflux transporter Yhhs in E. coli MG1655. Gene PA0243 encodes a putative type III TetR regulator without knowing its function and target genes. PA3808 has a similarity of 67.1% with IscX protein of ISC system FeS cluster assembly responsible for the ISC biogenesis in E. coli. In the absence of protein IscX, the sulfur flux isn’t allowed to be distributed to the U34 thiolation process of tRNA Gln, tRNA Lys, and tRNA. The modification of the tRNAs plays an important role in determining the ratio between protein gpG and gpGT of phage λ, and the ratio affects the efficiency of the assembly and release of phage λ progenies. Iron ion can be one of the key components of the functional phage tail like protein complexes. In phage λ, the tail tip complex (TTC) mediates the adsorption and injection of the genomic DNA during the infection process. The TTC protein Gp14 has four conserved Cys residues at its C-terminus for the binding of the ISC proteins in which iron ion is obtained via the protein IscX.

Materials and Methods
Bacterial strains, phage, plasmids, and growth conditions. The bacterial strains and the plasmids used in this study were listed in Table 2. Phage C11 was a virulent virus isolated from fresh water river samples using the clinical strain P. aeruginosa TJC422 as indicator. LB medium was used for the incubations of bacteria. If necessary, antibiotics with appropriate concentrations were added to the cultures, carbenicillin (150 μg/ml) and gentamicin (100 μg/ml) for P. aeruginosa incubations; ampicillin (100 μg/ml) and gentamicin (10 μg/ml) for E. coli incubations. All incubations were performed at 37 °C.

Characterization of phage C11. Phage particles of C11 for transmission electron microscopy (TEM) were prepared and purified as described previously. Briefly, the purified phage C11 solution was added on the carbon film over the copper grid for negative staining with 2% phosphotungstic acid (pH 6.7). Philips EM400ST transmission electron microscope was used for phage visualization. Purified phage particles were also subjected to SDS-PAGE for the viral structural proteins analysis. P. aeruginosa PAK was used as the indicator strain to determine the latent period and the burst size of phage C11 by one-step growth experiment as described previously at a MOI of 0.001. Triplicate cultures were involved in the experiment. Samples were diluted adequately and phage titers were measured by the double-layer plate method.
**Phage genome sequencing and identification of the terminal sequences.** The phage genomic DNA was extracted and purified for genome sequencing\(^6\). The whole genome sequencing was conducted by using the HiSeq Illumina 2500 System in GENEWIZ, Inc., China (http://www.genewiz.com/). The obtained sequences were filtered with the software Trimmomatic (v0.30) to remove the sequences with low quality and the adaptor sequences\(^6\). The total clean data included 6,983,460 reads with 692,093,907 bp and was assembled into a single contig with the software Velvet\(_{v1.2.10}\)\(^6\). The terminal identification was conducted according to the procedure described previously\(^61\). The HFS of the assembled genome which might represent the phage genome termini of were revealed by analyzing the sequencing depth across the genome\(^34\). The software DNAMAN was used to simulate the restriction mapping of the C11 genome on the basis of the termini inferred from the HFS sites. The *Prul* and *AIII* digested DNA fragments that possibly contained the termini of phage C11 were recovered from the electrophoresis gels, filled in with the large fragment (klenow), and ligated with T4 DNA ligase. The ligation products were amplified with the primers listed in Table 3, 3F and 3R for 3′ terminus determination and 5F and 5R for 5′ terminus determination. The amplicons were sequenced to determine the terminal sequences. The assembled genome sequence was curated and deposited in GenBank of NCBI with the accession number KT804923.

**Genome annotation and comparative genomic analysis.** The software tRNAscan-SE v1.2.1 was performed to predict the tRNA encoding genes\(^49\). Possible protein coding genes were predicted with DNA Master 5.0.2 (http://cobamide2.bio.pitt.edu/) and the threshold value was set at 100 bp. The annotation was carried out by searching against the non-redundant protein database (nr) from NCBI using the alignment software blast\_2.2.27\_\*\(^69\) and the tool provided at the website http://stothard.afns.ualberta.ca/cgview_server\(^70\). The genome sequences of phage PaP1 (NC\_019913), PAK\_P1 (NC\_015294), IG004 (NC\_019450), vB_PaeM\_C2-10\_Ab1 (NC\_019918), K5 (KU497559), and K8 (KT736033) in FASTA format were downloaded from the NCBI genome database for comparison analysis.

**Isolation and characterization of phage resistant mutants.** Tn5G transposon insertional library was constructed as described previously\(^71\). *E. coli* DH5α/pPK2013Tn5G and *P. aeruginosa* PAK were used as the donor strain and the recipient strain, respectively. The library was incubated with 5 ml phage C11 stock solution (1\(^{10}\) phi/ml) for 4h to enrich phage-resistant mutants. Phage-resistant mutants were screened on LB plates with gentamicin (100 \(\mu\)g/ml) and ampicillin (100 \(\mu\)g/ml). The adsorption rate of the isolated phage-resistant mutants was analyzed as previously described. Briefly, MOI of 0.001 was used in the test. After adsorption for 10 min, free phage particles were removed by centrifugation for titer analysis by the double-layer plate method\(^64\). To verify if the DAR mutants can resume their adsorption rates as strain PAK in the extended period, the time-course of adsorption rate was analyzed at a MOI of 0.0001. The cultures were sampled at 2 min intervals for further analysis of the free phage particles.

**Identification of the disrupted genes by inverse PCR.** To identify the insertional site of transposon Tn5G in the phage resistant mutants, inverse PCR method was performed as described\(^72\). Briefly, bacterial genomic DNA was digested completely by the restriction endonuclease *TaqI*. The digested DNA fragments were subsequently subjected to self-ligation. The ligated molecules were amplified using the primer pair OTn1 and OTn2 listed in Table 3. PCR products were sequenced and the obtained sequences were analyzed by searching the database GenBank of NCBI (http://www.ncbi.nlm.nih.gov/) and the *Pseudomonas* genome database (http://www.pseudomonas.com/).

**Complementation assay.** The genomic structure of the disrupted gene was analyzed by searching the website (http://www.pseudomonas.com/) and the promoter region of the genes were predicted in the website (http://fruitfly.org/seq_tools/promoter.html). The target genes for complementation tests were amplified by PCR using the primers listed in Table 3. The shuttle vector pUCP18 was used for the target gene cloning. Some genes were driven by their natural promoters and other genes located with operons were driven by the *Plac* promoter on the vector. The recombinant plasmids were transformed into the corresponding mutants by electroporation. The sensitivity of transformants to phage C11 was analyzed by the spotting assay in which 10\(^5\)–10\(^6\) phage particles were applied in each spot.

**Growth dynamic assay of the phage-resistant mutants.** The growth rate of the phage-resistant mutants was analyzed by incubation in 96-well plates in quadruplicate. At the beginning of incubation, phage C11 was added to the cultures at various MOIs (multiplicity of infection). The optical density of the cultures was measured at the wavelength of 600 nm.

**Real-time quantitative PCR analysis of phage genomic DNA.** The phage resistant mutants were infected with phage C11 according to the procedure previously used for one-step growth experiment at a MOI of 0.0001\(^6\). The samples were collected at 0 min and 30 min, respectively. Total DNA including phage genomic DNA was extracted as previously described\(^65\). The same amount of the purified DNA of each samples was used as templates for real-time quantitative PCR to measure the amount change of phage genomic DNA. *Go Taq* \(^q\)PCR Master Mix from Promega was used. Gene (*ORF056*) encoding RNA polymerase in phage C11 genome was chosen as the amplified target and the primers were designed (Table 3). *Pseudomonas* genomic DNA of strain PAK and water were used as negative controls in the analysis. The experiment was repeated at 3 times and each sample was analyzed in triplicate. The Ct (cycle threshold) value was used to evaluate the amount of the target templates.

**Southern blot analysis.** Bacterial culture of 40 ml was incubated to logarithmic phase (OD\(_{600}\) = 0.6). Harvested bacterial cells were resuspended in fresh LB medium and mixed with phage C11 at a MOI of 1. After
1 min adsorption, free phage particles in supernatant were removed by centrifugation, and cell pellet was transferred into 100 ml LB medium for incubation. Samples were removed at 30 min for total DNA preparation. The purified DNA was digested with the restriction enzyme ApaI, EcoRI, EcoRV, XhoI, and XbaI, respectively, and subjected to Southern blot analysis as described previously. The primers S1 and S2 were used to synthesize the probe of 520 bp for detecting the genomic DNA of phage C11 (Table 3 and Fig. S2A). The probe was labelled by using DIG High Prime DNA labeling and Detection Starter Kit II (Roche).

One unexpected 4.0 kb EcoRI band was detected in the Southern blot analysis. It’s predicted that one new EcoRI site was generated by spontaneous mutation in the C11 genome. To verify this hypothesis, the primers SM-F and S2 were used to amplify the 5′ terminal fragment and it was subsequently digested with EcoRI enzyme (Table 3 and Fig. S2). The 1.3 kb EcoRI fragment was purified from gel and sequenced directly to verify the existence of the new EcoRI site.

### Infection efficiency analysis in the WAR mutants.

The standard one-step growth experiment was performed as described previously with modifications. In brief, the same amount of phages and the same amount of host cells were used in all cultures at a MOI of 0.00001 in the test. After 1 min adsorption, free phage particles were removed by centrifugation and the pellet was resuspended in fresh LB medium. Repeat the step for three more times and the cell-phage complexes were finally resuspended in 100 ml medium. PAK strain was used as the indicator instead of the WAR mutants in the double-layer plating method to count the infection centres. The plaques were counted at 5 min and 35 min, respectively. The infection efficiency at 35 min was evaluated by comparing the phage titers of the WAR mutant cultures with that of the PAK cultures. The phage production of phage C11 was evaluated by comparing the titers at 5 min with that at 35 min. Triplicate cultures were involved in the experiment.

### Sensitivity testing with Pseudomonas aeruginosa phage K5 and K8.

The sensitivity of the isolated phage-resistant mutants to P. aeruginosa phage K5 and K8 were performed with the spotting assay method as described previously. The DNA probe for Southern blot analysis was labelled by using DIG High Prime DNA labeling and Detection Starter Kit II (Roche).

### References

1. Chan, B. K., Abedon, S. T. & Loc-Carrillo, C. Phage cocktails and the future of phage therapy. *Future microbiology* 8, 769–783, doi: 10.2217/fmb.13.47 (2013).
2. Kutter, E. *et al.* Phage therapy in clinical practice: treatment of human infections. *Current pharmaceutical biotechnology* 11, 69–86 (2010).
3. Chaturomgakul, S. & Ounjai, P. Phage-host interplay: examples from tailed phages and Gram-negative bacterial pathogens. *Frontiers in microbiology* 5, 442, doi: 10.3389/fmicb.2014.00442 (2014).

| Primer | *Primer sequence (5′-3′) | Target region |
|--------|--------------------------|---------------|
| OTn1   | GATCCCTGGAAACCGGAAAG     | genome DNA regions flanking by Tn5G |
| OTn2   | CCATCTCATGAGGGGTAGT      |               |
| BN889_05221-F | CCCAAGCTTGAAGGACGCTTCTTTGT | coding region of BN889_05221 |
| BN889_05221-R | CGCGGATCCACACCTTGCGTAGAATGTG |               |
| PA3808-F | CGCGGATCCGACAAACAGGCTTCA | coding region of gene PA3808 |
| PA3808-R | CGCGGATCCGACAAACAGGCTTCA |               |
| wbpR-F | CGCGGATCCGACAAACAGGCTTCA | gene wbpR with its natural promoter |
| wbpR-R | CGCGGATCCGACAAACAGGCTTCA |               |
| MPS-F   | CGCGGATCCGACAAACAGGCTTCA | gene PA1993 with its natural promoter |
| MPS-R   | CGCGGATCCGACAAACAGGCTTCA |               |
| PA5181-F | CGCGGATCCGACAAACAGGCTTCA | coding region of gene PA5181 |
| PA5181-R | CGCGGATCCGACAAACAGGCTTCA |               |
| PA0243-F | CCCAAGCTTCGCGGTGATGTCGGT | coding region of gene PA0243 |
| PA0243-R | CCCAAGCTTCGCGGTGATGTCGGT |               |
| PA1115-F | CCCAAGCTTCGCGGTGATGTCGGT | gene PA1115 with its own promoter |
| PA1115-R | CCCAAGCTTCGCGGTGATGTCGGT |               |
| S6-F    | GTGTTTGTCTCCTCCCGAGAG    | qPCR primers, coding region of gene ORF056 |
| S6-R    | ATTCGCTGTCCTTCCGCGG     |               |
| 3F      | TATCAGATGGAGGATGTTCA    | 3′ terminus of phage C11 genome |
| 3R      | GCTGAGAAGGACTTGGTC      |               |
| 5F      | GGTGTCCATCCACTCCGCTG    | 5′ terminus of phage C11 genome |
| 5R      | GTTGCGCTCTCGAGATAAT     | The DNA probe for Southern blot analysis |
| S1      | AGGCCCTGCTCTCGAGATCC    |               |
| S2      | TCCTAAGATGGGCTTGCCC     |               |
| SM-F    | ACACCTGAAAACCTTGAGCTCC  | Amplification of the fragment with a mutation generating an EcoRI site |
| S2      | TCCTAAGATGGGCTTGCCC     |               |

Table 3. Primers used in the study. The bases underlined represent the added restriction enzyme sites.
42. Bommer, D., Scharfjohann, J. & Bowien, B. Identification of cbbBc as an additional distal gene of the chromosomal cbb CO2 fixation operon from Raistonia eutropha. Archives of microbiology 166, 245–251 (1996).
43. Koita, K. & Rao, C. V. Identification and analysis of the putative pentose sugar efflux transporters in Escherichia coli. PLoS one 7, e43700, doi: 10.1371/journal.pone.0043700 (2012).
44. Krushkal, J. et al. Genotype diversity of the TetR family of transcriptional regulators in a metal-reducing bacterial family Geobacteraceae and other microbial species. Omics: a journal of integrative biology 15, 495–506, doi: 10.1089/omi.2010.0117 (2011).
45. Cutibertson, L. & Nodwell, J. R. The TetR family of regulators. Microbiology and molecular biology reviews: MMBR 77, 440–475, doi: 10.1128/MMBR.00018-13 (2013).
46. Beinert, H., Holm, R. H. & Munck, E. Iron-sulfur clusters: nature’s modular, multipurpose structures. Science 277, 653–659 (1997).
47. Pastore, C. et al. YfH, a molecular adaptor in iron-sulfur cluster formation or a frataxin-like protein? Structure 14, 857–867, doi: 10.1016/s0969-2126(06)00364-0 (2006).
48. Maynard, N. D., Macklin, D. N., Kirkegaard, K. & Covert, M. W. Competing pathways control host resistance to virus via tRNA modification and programmed ribosomal frameshifting. Molecular systems biology 8, 567, doi: 10.1038/msb.2011.101 (2012).
49. Kim, J. H., Bothe, J. R., Frederick, R. O., Holder, J. C. & Markley, J. L. Role of IscX in iron-sulfur cluster biogenesis in Escherichia coli. Journal of the American Chemical Society 136, 7933–7942, doi: 10.1021/ja501260h (2014).
50. Tam, W. et al. Tail tip proteins related to bacteriophage lambda gpl coordinate an iron-sulfur cluster. Journal of molecular biology 425, 2450–2462, doi: 10.1016/j.jmb.2013.03.032 (2013).
51. Bartual, S. G. et al. Structure of the bacteriophage T4 long tail fiber receptor-binding tip. Proceedings of the National Academy of Sciences of the United States of America 107, 20287–20292, doi: 10.1073/pnas.1112181107 (2010).
52. Yamashita, E. et al. The host-binding domain of the P2 phage tail spike reveals a trimeric iron-binding structure. Acta crystallographica. Section F, Structural biology and crystallographic communications 67, 837–841, doi: 10.1107/S1744309111005999 (2011).
53. Chelikani, V., Ranjan, T. & Kondabagil, K. Revisiting the genome packaging in viruses with lessons from the “Giants”. Virology 466–467, 15–26, doi: http://dx.doi.org/10.1016/j.virol.2014.06.022 (2014).
54. Oram, M. & Black, L. W. In Structural Virology 203–219 (The Royal Society of Chemistry, 2011).
55. Sun, S., Rao, V. B. & Rossmann, M. G. Genome packaging in viruses. Current opinion in structural biology 20, 114–120, doi: 10.1016/j.sbi.2009.12.006 (2010).
56. Oliveira, L., Tavares, P. & Alonso, I. C. Headful DNA packaging: Bacteriophage SPP1 as a model system. Virus research 173, 247–259, doi: http://dx.doi.org/10.1016/j.virusres.2013.01.021 (2013).
57. Leffers, G. & Basaveswara Rao, V. A Discontinuous Headful Packaging Model for Packaging Less Than Headful Length DNA Molecules by Bacteriophage T4. Journal of molecular biology 258, 839–850, doi: http://dx.doi.org/10.1016/j.jmb.1996.0291 (1996).
58. Nurnemmedovod, E., Castelnovo, M., Medina, E., Catalano, C. E. & Evlivić, V. Challenging Packaging Limits and Infectivity of Phage . Journal of molecular biology 415, 263–273, doi: http://dx.doi.org/10.1016/j.jmb.2011.11.015 (2012).
59. Drake, J. W. A constant rate of spontaneous mutation in DNA-based microbes. Proceedings of the National Academy of Sciences of the United States of America 88, 7160–7164 (1991).
60. Sanjuan, R., Nebot, M. R., Chirico, N., Mansky, L. M. & Belshaw, R. Viral mutation rates. Journal of virology 84, 9733–9748, doi: 10.1128/JVI.00694-10 (2010).
61. Pan, X. et al. Genetic Evidence for O-Specific Antigen as Receptor of Pseudomonas aeruginosa Phage K8 and Its Genomic Analysis. Frontiers in microbiology 7, doi: 10.3389/fmicb.2016.00252 (2016).
62. Li, L. et al. Characterization of Pseudomonas aeruginosa phage K5 genome and identification of its receptor related genes. J. Basic Microbiol. 56, 1–11, doi: 10.1002/jobm.201600116 (2016).
63. Li, L., Yang, H., Lin, S. & Jia, S. Classification of 17 newly isolated virulent bacteriophages of Pseudomonas aeruginosa. Canadian journal of microbiology 56, 925–933, doi: 10.1139/w10-075 (2010).
64. Yang, H., Liang, L., Lin, S. & Jia, S. Isolation and characterization of a virulent bacteriophage AB1 of Acinetobacter baumannii. BMC microbiology 10, 131, doi: 10.1186/1471-2180-10-131 (2010).
65. Sambrook, J. Molecular cloning: a laboratory manual/Joseph Sambrook, David W. Russell. (Cold Spring Harbor Laboratory, 2001).
66. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120, doi: 10.1093/bioinformatics/btu170 (2014).
67. Zerbino, D. R. Using the Velvet de novo assembler for short-read sequencing technologies. Current protocols in bioinformatics/ editorial board, Andreas D. Baezeman … [et al.] Chapter 11, Unit 11 15, doi: 10.1002/0471250953.bi1105s31 (2010).
68. Lowe, T. M. & Eddy, S. R. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic acids research 25, 953–964 (1997).
69. Altschul, S. F. et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic acids research 25, 3389–3402 (1997).
70. Grant, J. R. & Stothard, P. The CGView Server: a comparative genomics tool for circular genomes. Nucleic acids research 36, W181–184, doi: 10.1093/nar/gkn179 (2008).
71. Nunn, D. N. & Lory, S. Components of the protein-excretion apparatus of Pseudomonas aeruginosa are processed by the type IV prepilin peptidase. Proceedings of the National Academy of Sciences of the United States of America 89, 47–51 (1992).
72. Vallet, I., Olson, J. W., Lory, S., Lazdunski, A. & Filloux, A. The chaperone/usher pathways of Pseudomonas aeruginosa: identification of fimbrial gene clusters (cup) and their involvement in biofilm formation. Proceedings of the National Academy of Sciences of the United States of America 98, 6911–6916, doi: 10.1073/pnas.111551898 (2001).
73. Bradley, T. I. & Khan, N. H. The production of extracellular lipids by Pseudomonas Aeruginosa NCTC 2000 in stationary liquid media containing macrogols. The journal of pharmacy and pharmacology 26, 900–902 (1974).
74. Schweizer, H. P. Escherichia-Pseudomonas shuttle vectors derived from PUC18/19. Gene 97, 109–121 (1991).

Acknowledgements
This work is supported by The National Natural Science Foundation of China (Grant No. 31370205 and 30970114).

Author Contributions
X.C. performed the bioinformatic analysis and experiments and wrote the manuscript. J.Y. and L.S. contributed to Fig. 7. X.Y., T.Z., K.H., X.P. and F.Z. carried out the plasmid constructions. Y.H. performed the bioinformatic analysis. H.Y. designed the experiments and wrote the manuscript. All authors reviewed the manuscript.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.
