Prophage encoding toxin/antitoxin system PfIT/PfIA inhibits Pf4 production in Pseudomonas aeruginosa

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Summary

Pf prophages are ssDNA filamentous prophages that are prevalent among various Pseudomonas aeruginosa strains. The genomes of Pf prophages contain not only core genes encoding functions involved in phage replication, structure and assembly but also accessory genes. By studying the accessory genes in the Pf4 prophage in P. aeruginosa PAO1, we provided experimental evidence to demonstrate that PA0729 and the upstream ORF Rorf0727 near the right attachment site of Pf4 form a type II toxin/antitoxin (TA) pair. Importantly, we found that the deletion of the toxin gene PA0729 greatly increased Pf4 phage production. We thus suggest the toxin PA0729 be named PfIT for Pf4 inhibition toxin and Rorf0727 be named PfIA for PfIT antitoxin. The PfIT toxin directly binds to PfIA and functions as a corepressor of PfIA for the TA operon. The PfIA-T complex exhibited autoregulation by binding to a palindrome (5′-AATCNGTTAA-3′) overlapping the -35 region of the TA operon. The deletion of pfIT disrupted TA autoregulation and activated pfIA expression. Additionally, the deletion of pfIT also activated the expression of the replication initiation factor gene PA0727. Moreover, the Pf4 phage released from the pfIT deletion mutant overcame the immunity provided by the phage repressor Pf4r. Therefore, this study reveals that the TA systems in Pf prophages can regulate phage production and phage immunity, providing new insights into the function of TAs in mobile genetic elements.

Introduction

Toxin/antitoxin (TA) systems are genetic modules widely distributed in prokaryotes. TA genes usually encode a toxin that kills cells or inhibits cell growth and a cognate antitoxin that neutralizes the toxicity of the toxin. A total of six types of TA systems have been identified based on the molecular features (protein or RNA) of antitoxins and the mechanisms they used to mask the toxicity of toxins (Mruk and Kobayashi, 2014). In type II TA systems, both toxins and antitoxins are proteins, and antitoxins neutralize the toxicity of toxins by direct protein–protein interactions. Toxin and antitoxin genes are in the same operon, and the cognate toxins either work as repressors or activators of antitoxins to autoregulate the expression of the TA operon (Magnuson and Yarmolinsky, 1998; Affif et al., 2001; Overgaard et al., 2008; Winther and Gerdes, 2012; Turnbull and Gerdes, 2017). These type II TA systems are found in both chromosomes and mobile genetic elements including plasmids and prophages (Wang and Wood, 2016; Harms et al., 2018). Studies of TA systems in plasmids are more extensive than those in prophages. The studied plasmid-encoded TA systems include the first type II TA CcdB/CcdA characterized ‘addiction’ systems on the F sex factor plasmid (Ogura and Hiraga, 1983), ParE/ParD, Hk/Doc, HigB/HigA and HicB/HicA (Lehnerr et al., 1993; Roberts et al., 1994; Hayes, 2003; Christiansen-Dalsgaard and Gerdes, 2006; Kroll et al., 2010).

Prophages and satellite prophages are some of the major horizontal gene transfer elements that are widespread among bacteria, and they constitute up to 20% of bacterial genomes. Many sequenced bacterial genomes contain multiple prophages, e.g. eighteen prophages...
were identified in *E. coli* O157 Sakai (Asadulghani et al., 2009), and nine prophages were identified in *E. coli* K12 MG1655 (Wang et al., 2010). Prophages confer a series of phenotypic traits to their hosts, including pathogenicity (Sweere et al., 2019), antibiotic tolerance and resistance (Wang and Wood, 2016), biofilm formation and general stress (Wang et al., 2010; Wang and Wood, 2011; Zeng et al., 2016). The genomes of most prophages not only contain genes encoding functions involved with phage replication, structure and assembly, but also contain accessory genes. For example, the well-characterized MG1655 prophages encode type I, type II and type IV toxin/antitoxin (TA) systems. In particular, the product of toxin *railR* in the rac prophage is a DNase, and the type I RelR/RelA TA pair increased cell resistance to fosfomycin (Guo et al., 2014). In addition, the type IV TA pair CbtA/CbeA in the cryptic prophage CP4-44 has been related to resistance to norfloxacin, novobiocin and spectinomycin (Kohanski et al., 2007; Masuda et al., 2012). In *Shewanella oneidensis*, a type II TA pair ParESO/CopASO in the cryptic prophage CP4So stabilizes the circular prophage CP4So in host cells after its excision (Yao et al., 2018). In addition, infection of lytic phages is also inhibited by plasmid- or chromosomal-encoded TA systems. The type I TA system Hok/Sok from plasmid R1 excludes T4 infection in *E. coli* (Pecota and Wood, 1996), and the chromosomal type II TA system MazE/MazF protects cells from P1 phage infection (Hazen and Engelberg-Kulka, 2004). In addition, the first type III TA system, ToxN/ToxI, was found in a cryptic plasmid of the plant pathogen *Pectobacterium atrosepticum* that supplies cells with an ability to resist to other phages by the release of the ribonuclease toxin ToxN (Fineran et al., 2009).

*Pseudomonas aeruginosa* is an opportunistic pathogen found to infect plants, invertebrates and vertebrates (Palleroni, 1984) and is clinically important for chronic lung infections in cystic fibrosis (CF) patients (Lyczak et al., 2002). These *P. aeruginosa* strains frequently contain prophages, and prophages are important in the CF-epidemic strains. The filamentous phage PfI is critical for several stages of the *P. aeruginosa* biofilm life cycle (Rice et al., 2009; Secor et al., 2015) and is a key contributor to the formation of small colony variants and virulence in vivo (Ilyina, 2015; Sweere et al., 2019). Three putative TA loci have been predicted in the genome of the model strain *P. aeruginosa* PAO1 by bioinformatic analysis (Williams et al., 2011), and HigB/HigA on the chromosome was shown to be a type II TA system that controls biofilm formation and virulence (Li et al., 2016, Wood and Wood, 2016; Zhang et al., 2018, Guo et al., 2019). In the present study, we characterized the type II TA system PfIT/PfIA in the PfI4 prophage of PAO1 and found that it controls the production of the PfI4 phage. PfIT greatly inhibits cell growth, and PfIA neutralizes the toxicity of PfIT through direct protein–protein interactions. The PfIA and PfIT genes are cotranscribed and the PfI4AT complex, but not antitoxin PfIA, autoregulates the TA operon by binding to the palindrome 5’-AATTCCN5-GT-TAA-3’, overlapping the –35 region of the TA operon. The deletion of the toxin *pfIT* gene induced the production of PfI4 phage by increasing the expression of the replication initiation factor gene, and the phages released from the toxin *pfIT*-deleted strain can overcome the immunity supplied by the phage repressor Pf4r. To the best of our knowledge, this is the first experimental evidence that a TA system in a filamentous phage controls phage production.

**Results**

*PfIT* and *PfIA* in the PfI4 prophage form a TA pair

We recently reannotated the PfI4 genome during the identification of the phage excisionase gene *xisF4* (Li et al., 2019). Two neighbouring genes that are only 9 bp apart, PA0729 and Rorf0727, are located at the right end of the PfI4 prophage. *Rorf0727* encodes a protein of 83 aa that belongs to the Phd antitoxin family (here, we renamed it PfIA), and PA0729 encodes a protein of 115 aa that belongs to the ParE toxin family (here, we renamed it PfIT; Fig. 1A). To determine whether they constitute a *bona fide* TA pair, open reading frames of the two genes were cloned into plasmid pMQ70 to obtain pMQ70-pfIA and pMQ70-pfIT, respectively, using the primers listed in Table S1. Expression of *pfIT* or *pfIA* was induced in PAO1 with 10 mM L-arabinose. Cell growth (turbidity) and cell viability (CFU ml⁻¹) were measured over time. Overexpression of *pfIT* in PAO1 led to not only growth inhibition but also cell death (Fig. 1B, C). In contrast, overexpression of *pfIA* did not affect cell growth or cell death. To further assess whether PfIA can block the toxicity of PfIT, we cloned the coding region of *pfIA* and *pfIT* to construct pMQ70-pfIA-pfIT, which was used to coexpress *pfIA* and *pfIT* in PAO1. Coexpression of *pfIT* with *pfIA* showed similar growth and cell viability compared with the empty vector pMQ70 (Fig. 1B, C), indicating that PfIA neutralized PfIT toxicity. Thus, PfIA functions as an antitoxin to prevent the growth-inhibitory effect of toxin PfIT. Since most TA systems are cotranscribed, we then conducted a primer extension assay using the oligonucleotide FAM-pfIT-r (Table S1), which is complementary to *pfIT*, to search for the transcription start of the TA operon. As shown in Fig. 1D, the major extension product is 700 nt in size, indicating that *pfIA* and *pfIT* are cotranscribed and that the transcriptional start site of the *pfIAT* operon is 127 bp upstream of *pfIA* (Fig. 1A, D). Collectively, these results demonstrated that the antitoxin PfIA and toxin PfIT form a type II TA pair.

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**PfiA interacts with PfiT in vivo**

For most type II TA systems, the toxin interacts with the antitoxin directly to form a protein complex in vivo. To test whether PfiA binds to PfiT, a pull-down assay was performed with pET28b-PfiAT-His to coexpress a C-terminal hexahistidine-tagged (His-tagged) PfiT with untagged antitoxin PfiA. As expected, affinity purification revealed that another protein was pulled down along with His-tagged PfiT (expected size ~ 13.81 kDa) using Ni-NTA agarose beads and subsequent tricine-SDS-PAGE (Fig. 2A), and the size of this protein was consistent with the size of the PfiA antitoxin (~ 9.44 kDa). To further determine the interaction between PfiA and PfiT, a bacterial two-hybrid (BATCH) assay based on the physical interaction of the T18 and T25 catalytic domains...
was conducted. An in-frame translational fusion between the T18 catalytic domain and pfiA was performed to generate pUT18C-pfiA, and a similar fusion between the T25 catalytic domain and pfiT was also generated (pKT25-pfiT). For the positive control, a fragment encoding a 35 aa leucine zipper was translationally fused to the T25 and T18 catalytic domains to generate pKT25-zip and pUT18C-zip respectively. For the negative control, the empty vector pKT25 without an insert and pUT18C-zip were used. Consistent with the above pull-down assay, pKT25-pfiT and pUT18C-pfiA showed clear β-galactosidase activity, indicating that the interaction between PfiA and PfiT occurred (Fig. 2B). Taken together, PfiA and PfiT form a complex in vivo, and the inhibitory effect of PfiA to PfiT is likely due to the direct interaction between them.

The PfiAT complex controls pfiAT transcription

Type II antitoxins alone or in complex with toxins can bind to their promoters and negatively regulate the transcription of the TA operon. To test whether PfiA affects TA promoter activity, we transcriptionally fused a 254 bp

promoter region to lacZ and integrated it into the chromosome of PAO1 via a mini-CTX plasmid according to a previously reported method (Hoang et al., 2000). We also constructed TA deletion mutants in PAO1. Two mutants, ΔpfiT and ΔpfiAT, were constructed and confirmed by PCR and DNA sequencing (Fig. 3A). We tried to knock out pfiA in this experiment, but no correct strain was obtained after extensive effort, indicating that this antitoxin may not be able to be removed due to the strong toxicity of the toxin. Then, the promoter activity was determined in the PAO1 wild-type strain and the two deletion mutant strains. The β-galactosidase activity in PAO1 wild-type cells was 185.51 ± 15.54 Miller units (MU), and it increased to 662.82 ± 15.93 MU in the ΔpfiAT cells (Fig. 3B). These results showed that PfiT/ PfiA negatively regulates its own promoter activity. However, there was no significant change in the promoter activity between the ΔpfiT and ΔpfiAT cells (664.73 ± 46.18 MU versus 662.82 ± 15.93), suggesting that antitoxin PfiA alone may not be sufficient for the autoregulation of the TA operon. To further investigate this, PfiA and the PfiAT TA complex were produced via pHerd20T-pfiA and pHerd20T-pfiAT in the two deletion mutant reporter strains, and the promoter activity was determined. Consistent with the above results, only a slight decrease in β-galactosidase activity was observed when PfiA was overexpressed compared with the empty vector in both reporter strains. However, a significant decrease in β-galactosidase activity was observed when the PfiAT complex was coexpressed compared with the empty vector (Fig. 3C). In addition, the autoregulation of the PfiT/PfiA TA pair was determined with EMSA. A PCR product of 254 bp, which included the promoter region of the TA operon, was used to bind with PfiA or the PfiAT complex. The PfiAT complex specifically bound to the pfiAT promoter region (Fig. 3D, lanes 1–4). However, no binding to the promoter region was observed for PfiA in the absence of the toxin (Fig. 3D, lanes 8–10), and the binding appeared when the PfiAT TA complex was added (Fig. 3D, lanes 5–7). Thus, the PfiAT complex represses the transcription of the pfiAT operon by binding to the TA promoter region, and PfiT functions as a corepressor of PfiA.

The PfiAT complex binds to 5’-AATTGAGAATT-3’ in the pfiAT promoter

Bioinformatics analysis of the pfiAT operon identified a palindromic sequence, 5’-AATTGAGAATT-3’, overlapping the predicted−35 region of pfiAT (Fig. 1A). To determine the exact binding site of the PfiAT complex, a DNase I footprinting assay was employed using the 300 bp promoter region of pfiAT and the purified
PiFIAT complex. The results showed that the region containing the palindrome was specifically protected from DNase I digestion by the PiFIAT complex (Fig. 4A). To further confirm the DNA-binding ability of the PiFIAT complex to the palindrome in vivo, we constructed a series of lacZ reporter plasmids with different mutations in the palindromic sequence. Plasmids pLP170-M1-πFIAT and pLP170-M2-πFIAT contain one mutation each in the left arm and right arm, respectively, and pLP170-M3-πFIAT contains mutations in both arms (Fig. 4B). Then, the β-galactosidase activities were determined in both PAO1 wild-type and ΔπFIAT strains. All mutations in these constructs increased the β-galactosidase activities significantly in PAO1 cells, which contain the πFIAT operon, indicating that the palindromic sequence is critical for the promoter activity of the πFIAT operon. In addition, mutation of the palindromic sequence had no effect on β-galactosidase activity in ΔπFIAT cells, showing that the PiFIAT complex binds to the palindromic sequence. Taken together, the PiFIAT complex represses its own expression by binding to 5'–AAATCNGAATT–3' in the πFIAT promoter.

PiFIAT inhibits Pf4 replication by inducing PA0727

To probe the physiological function of the PiFIAT TA pair, we investigated Pf4 production by the deletion mutant ΔπIT. Specifically, wild-type PAO1 and ΔπIT cells were cultured statically in LB medium to form pellicle biofilms, and the supernatant was collected at different time points to determine the plaque-forming units (PFU) in the Pf4 deletion strain (ΔPf4). As shown in Fig. 5A, deletion of πIT greatly increased Pf4 phage production over time (left) and increased Pf4 phage production by approximately 100,000-fold compared with the wild-type at 6 h (right). To explore how PiIA regulates Pf4 phage
production, qRT-PCR was used to quantify the expression of Pf4 genes in the wild-type and ΔpfiT strains. The amplification efficiencies of the primer sets used in qRT-PCR lie between 89.3 and 106.7% (Fig. S1). Since the autorepression of the TA pair was disrupted in the ΔpfiT strain, as expected, we found that the expression of anti-toxin pfiA was induced 19.68/C6 4.15-fold when pfiT was deleted (Fig. 5B). In addition, the phage excisionase coding gene xisF4 and replication initiation protein-coding gene PA0727 were induced 71.00/C6 0.52-fold and 16.23/C6 1.67-fold, respectively, when pfiT was deleted, but not the phage repressor coding gene pfi4r (Fig. 5B). However, the excision of the Pf4 prophage was not induced in the ΔpfiT cells (data not shown). Therefore, disruption of the cooperativity of PfiA and PfiT induced the replication of the Pf4 phage by inducing PA0727 expression, thus increasing phage production.

**PfiT coordinates Pfi4r in conferring immunity to Pf4**

We have found that the phage repressor Pfi4r confers immunity to Pf4 (Li et al., 2019). To test whether infection of the phages from the wild-type PAO1 and ΔpfiT cells is both inhibited by Pfi4r, the production of Pf4 was induced by overexpressing XisF4 via pH2RD20T-xisF4 in wild-type PAO1 and ΔpfiT hosts. Similar phage titres of phages were obtained from supernatant of the two strains after induction with 10 mM arabinose for 4 h (Fig. 6 left panel). Then, the Pf4 phages were used to infect the ΔPf4 strain with overexpressing pfi4r. Consistent with our earlier work, overexpression of pfi4r in the ΔPf4 host strain provided higher immunity (∼10,000-fold higher) than the empty vector for the Pf4 phage released from wild-type PAO1. In contrast, the immunity against phage infection was greatly reduced for the phage released from ΔpfiT cells, approximately 10-fold higher than the empty vector. This result suggested that PfiT is involved in both phage production and phage immunity.

**Discussion**

In this study, we provided evidences that the Pf4 prophage encoded type II TA pair controls the production of filamentous phages in PAO1. The results were as follows: (i) PfiT is a toxin, and its toxicity can be neutralized by its cognate antitoxin PfiA; (ii) PfiA and PfiT interact with each other directly, and the PfiAT complex binds to the 5′-AATTCN5′GAATT-3′ palindrome in the pfiAT operon; (iii) mutation of pfiT increases production of Pf4 phage by inducing the expression of the replication initiation protein; and (iv) PfiT coordinates Pfi4r in conferring immunity to Pf4.
immunity to Pf4. Therefore, we proved that the toxin harboured in the prophage inhibits filamentous phage production in *P. aeruginosa*, and it also contributes to cell immunity to Pf4 phage infection, extending the physiological roles of the type II TA system.

A schematic of our understanding of how the PfT/PfA TA system controls the production of the Pf4 phage and further affects virulence and biofilm formation in PAO1 is shown in Fig. 7. In certain typical type II TA systems, the antitoxins act as transcriptional repressors and adopt N-terminal DNA-binding domains such as helix–turn–helix, ribbon–helix–ribbon and AbrB-type domains (Chan et al., 2016). However, PfA has a truncated N-terminus without these domains, which is similar to other Phd family antitoxins. The binding of the Phd family antitoxins to target sites requires the help of a toxin (Guerout et al., 2013), and a fully folded conformation where all secondary structure elements are formed after binding to the toxin (Cherny and Gazit, 2004; Garcia-Pino et al., 2010). Here, we found that the binding of PfA to the TA promoter region also requires PfT. The binding of PfT to the PfA antitoxin may stabilize the N-terminal domain, and change the allosteric and intrinsic disorder and thus control transcription regulation. A similar mechanism was observed in different TA systems, including Doc/Phd, CcdB/CcdA, RelE/RelB and VapC/VapB (Magnuson and Yarmolinsky, 1998; Afif et al., 2001; Overgaard et al., 2008; Winther and Gerdes, 2012). In some other TA systems, such as HigB/HiA and HicB/HicA, toxins are repressors of antitoxins and function in the transcriptional repression of the TA operon (Turnbull and Gerdes, 2017; Guo et al., 2019). ParE family toxins function as gyrase inhibitors and inhibit cell division by targeting GyrB (Jiang et al., 2002). We did not observe aberrant cell division when *pf*T was overexpressed in PAO1,
Filamentous phages are considered some of the simplest life forms on earth, and they have relatively smaller genomes (7–12 kb) compared with dsDNA tailed phage. Filamentous phages such as Pf4 and Pf5 are integrated as prophages in the genomes of PAO1 and PA14 respectively. In PAO1, Pf4 is integrated between PA0714 and PA0728, while Pf5 is integrated inside the coding region of the PA14_49040 gene. The genome regions that encode phage replication, structure and assembly genes in the prophages Pf4 and Pf5 share much higher sequence identity than accessory gene regions (Li et al., 2019). The PfIT/PfIA TA system is located at the end of the Pf4 prophage and is not found in the Pf5 prophage. A unique feature of the Pf4 phage in PAO1 is the ability to cause superinfection (Rice et al., 2009), and no superinfection was reported for the Pf5 phage in PA14. In this study, we found that the Pf4 phage released from the toxin pfIT-deleted strain can still efficiently infect PAO1 cells overexpressing the phage repressor gene pf4r. Although no similar palindromic sequence was identified in other regions of the Pf4 genome, the expression levels of the excisionase gene xisF4 and replication initiation factor PA0727 were induced significantly in the pfIT mutant strain, while no change in the expression level of the repressor gene pf4r was observed.

TA systems have a broad and important impact on bacterial physiology and bacterial pathogenicity by influencing developmental cascades such as the switch from planktonic to biofilm cells and/or the activation of the expression of virulence genes (Guo et al., 2019). Pf4 phage production was mainly found during PAO1 biofilm formation. Here, we found that the type II TA system PfIT/PfIA encoded by Pf4 controls Pf4 production, as the deletion of the toxin induces the production of Pf4. Indeed, the ratio between the toxin and the antitoxin was greatly changed in the PAO1 WT and in the pfIT deletion mutant strains, as the deletion of pfIT also induced the expression of pfIA. Under conditions when more PfIA is present, PfIA is likely to induce Pf4 replication. On the other hand, most type II antitoxins are usually unstable. Under specific conditions, PfIA can be degraded by certain proteases to free PfIT. Accordingly, free PfIT is able to inhibit the replication of the phage. Thus, the ratio between the toxin and the antitoxin seems important in the regulation of Pf4 production. This could either enable the bacterial host cells to control Pf4 production or equip the phage to trigger its own replication when needed. However, there are still unsolved questions that need to be addressed to obtain a better understanding of the role of the TA system in controlling phage production during biofilm formation.

**Experimental procedures**

**Bacterial strains, plasmids and growth conditions**

Bacterial strains and plasmids are listed in Table 1, and primers are listed in Table S1. E. coli and P. aeruginosa PAO1 strains were grown in Luria–Bertani (LB) medium at 37°C. Cells harbouring plasmids with the indicated resistance genes were cultured in medium supplemented with the following antibiotics at the indicated concentrations: kanamycin (50 μg ml⁻¹), tetracycline (50 μg ml⁻¹), gentamycin (30 μg ml⁻¹) and carbenicillin (100 μg ml⁻¹).

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### Table 1. Bacterial strains and plasmids used in this study.

| Strains/plasmids | Description | Source |
|------------------|-------------|--------|
| DH5α             | F−80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(k− m−) F− supE44 thi-1 gyrA96 relA1 tonA1 mcrB1 | Novagen |
| BTH101           | F−, cya-99, araD139, galK16, rpsL1 (Str'), hsdR2, mcrB1 | Euromedex Kit |
| PAO1             | Wild-type | Stover et al. (2000) |
| ΔP4              | Whole P4 prophage removed from PAO1 host chromosome | Li et al. (2019) |
| ΔpIT             | pIT deletion mutant derived from PAO1 chromosome | This study |
| ΔpIA             | pIA deletion mutant derived from PAO1 chromosome | This study |
| PAO1::pGAT-lacZ  | LacZ reporter strain | This study |
| ΔpIT::pGAT-lacZ  | LacZ reporter strain | This study |
| ΔpIA::pGAT-lacZ  | LacZ reporter strain | This study |

#### Plasmids

| Plasmids | Source |
|----------|--------|
| pET28b   | KmR, expression vector | Novagen |
| pET28b-pIA | KmR, pIA in pET28b Ncol/Hind III | This study |
| pET28b-pIAAT | KmR, pIAAT in pET28b Ncol/Hind III | This study |
| pMQ70    | ApR, CarR, expression vector with ara-C-PBAD promoter | Shanks et al. (2006) |
| pMQ70-pIA | ApR, CarR, pIA in pMQ70 SacI/Kpnl | This study |
| pMQ70-pIT | ApR, CarR, pIT in pMQ70 SacI/Kpnl | This study |
| pMQ70-pIAAT | ApR, CarR, pIAAT in pMQ70 SacI/Kpnl | This study |
| pHED20T   | ApR, CarR, expression vector with ara-C-PBAD promoter | Qiu et al. (2008) |
| pHED20T-pIA | ApR, CarR, pIA in pHED20T Ncol/sall | This study |
| pHED20T-pIAAT | ApR, CarR, pIAAT in pHED20T EcoRI/HindIII | This study |
| pHED20T-pIT | ApR, CarR, xisF in pHED20T Ncol/HindIII | Li et al. (2019) |
| pKT25-zip | KmR, derived from pKT25. Sequence coding for the leucine zipper region of the GCN4 yeast protein. Positive control | Karimova et al. (1998) |
| pKT25-pIT | KmR, expression vector for pIT. | This study |
| pUT18C    | ApR, derived from pUC19. Plac−-MCS(HindIII–SphI–PstI–SalI–XbaI–BamHI–Smal–KpnI–SacI–EcoRI–T18 | Karimova et al. (1998) |
| pUT18C-zip | ApR, derived from pUC19. Sequence coding for the leucine zipper region of the GCN4 yeast protein. Positive control. | Karimova et al. (1998) |
| pEX18AP   | ApR, onT , sacB , gene replacement vector | Hoang et al. (1998) |
| pLP12     | ApR, Flp recombinase-expressing plasmid | Hoang et al. (1998) |
| pPS856    | ApR, GmR; for amplifying gentamicin resistance cassette | Hoang et al. (1998) |
| pEX18AP-pIT | GmR, CarR, for deleting pIT | This study |
| pEX18AP-pIAAT | GmR, CarR, for deleting pIAAT | This study |
| mini-CTX-LacZ | TetR, integration vector for single-copy, chromosomal lacZ fusions; Ω-FRT-att-P-MCS, ori, int, and onT | Becher and Schweizer (2000) |
| pCTX-PGAT-lacZ | TetR, −313 bp relative to translational start site of pIT cloned into mini-CTX-lacZ | This study |
| pLP170    | CarR, promoterless-lacZ | Pesci et al. (1997) |
| pLP170-pIT | Wild-type promoter of pITAT fused into the lacZ of pLP170 | This study |
| pLP170-M1-pIAAT | FP1 mutant promoter of pIAAT fused into the lacZ of pLP170 | This study |
| pLP170-M2-pIAAT | FP3 mutant promoter of pIAAT fused into the lacZ of pLP170 | This study |
| pLP170-M3-pIT | FP4 mutant promoter of pITAT fused into the lacZ of pLP170 | This study |

#### Construction of deletion mutants in PAO1

The gene deletion strain was constructed as described previously in *P. aeruginosa* (Hoang et al., 1998). Briefly, the upstream and downstream regions of pIT and pIAAT were amplified through PCR from PAO1 genomic DNA. The gentamicin resistance gene cassette was amplified through PCR from the plasmid pPS856. These three amplicons were then ligated into the suicide plasmid pEX18Ap using the ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China). In-frame deletion mutants were obtained via homologous recombination using the sucrose resistance selection method. The gentamicin resistance cassette was removed from the chromosome as described previously (Hoang et al., 1998). Finally, the correct mutants were confirmed by PCR and DNA sequencing.

#### Construction of reporter strains

The full coding regions of pIA, pIT and pIAAT were PCR-amplified from PAO1 genomic DNA, and the PCR products were purified and ligated into the vectors pMQ70, pHERD20T and pET28b using the Vazyme ClonExpress II One Step Cloning Kit. For construction of promoter–reporter strains, the 254 bp upstream of pIAAT...
was amplified by PCR and ligated into the plasmid mini-
CTX-lacZ. The correct plasmids were transformed into
the PAO1, ΔpfiT and ΔpfiAT hosts and integrated into
chromosomes at the attB site near the tRNA^Ser
sequence using a previously described method (Becher
and Schweizer, 2000). Then, the tetracycline selection
marker was removed as described (Hoang et al., 1998).

β-Galactosidase activity assay

Specific β-galactosidase activities of strains PAO1, ΔpfiT
and ΔpfiAT harbouring the pfiAT promoter were deter-
mined by monitoring the absorbance at 420 nm using a
Pro200 Multi-Detection Microplate Reader (Tecan,
Männedorf, Switzerland) using the Miller assay method
(Miller, 1972). Overnight cultures were diluted 100-fold in
LB with or without carbenicillin (50 μg ml⁻¹) and grown
at 37 °C to an OD₆₀₀ of 1.0, and then, β-galactosidase
activity was determined. To determine the promoter
activity of pfiAT in ΔpfiT and ΔpfiAT carrying
pHERD20T-derived plasmids, overnight strains were
diluted to OD₆₀₀ ~ 0.1 and grown in LB supplemented
with carbenicillin and 10 mM arabinose. After induction
for 3 h, cells were collected to determine β-galactosidase
activity.

Bacterial two-hybrid (BACTH) assay

The BACTH assay was conducted as described (Battesti
and Bouveret, 2012) to investigate the interaction
between PfiA and PfiT in vivo. The coding regions of
pfiA and pfiT were cloned into pUT18C and pKT25
respectively. The recombinant plasmids were cotrans-
formed into E. coli BTH101 (cya-99) competent cells with
selection for kanamycin and ampicillin resistance. Then,
10 μl of overnight culture was spotted on LB plates sup-
plemented with kanamycin, ampicillin, IPTG (1 mM) and
X-gal (40 μg ml⁻¹). The colonies grew for 20 h. The
negative and positive controls were included as we
described previously (Yao et al., 2018).

Primer extension

The 5’-end FAM (6-carboxyfluorescein)-labelled primer
FAM-pfiT-r was ordered from Invitrogen (Carlsbad, CA,
USA). Total RNA was isolated from PAO1 wild-type
cells. The extension reactions were carried out with
10 μg of total RNA, 2 × 10⁻⁴ pmol of FAM-pfiT-r and
37.5 U of AMV reverse transcriptase (Promega, Madi-
son, USA). The reaction mixture was incubated at 42°C
for 90 min, and the products were concentrated with
centrifugal filter units (Millipore, Bedford, MA, USA)
before being loaded into an ABI3730 DNA Analyzer
(Applied Biosystems, Foster City, CA, USA).

Protein purification

Proteins PfiA and PfiAT were purified from the E. coli
BL21 (DE3) strain containing plasmid pET28b-pfiA or
pET28b-pfiAT respectively. One litre of LB supplemen-
ted with kanamycin was inoculated with 10 ml of overnight
culture, and the bacteria were grown with shaking at
37°C. IPTG 0.5 mM was added at OD₆₀₀ 0.5, and all the
cells were collected by centrifugation after induction for
6 h. The subsequent steps of protein extraction from the
collected pellet were performed as previously described
(Liu et al., 2015).

Electrophoretic mobility shift assay (EMSA)

The DNA probe of the promoter region of pfiAT was
amplified from the genomic DNA of the PAO1 strain
using the primer pair pfiAT-promoter-f/r (Table S1). The
purified DNA fragments were labelled with biotin by using
the Biotin 3’-End DNA Labeling Kit (Thermo Scientific,
Rockford, USA). Then, the biotin-labelled DNA fragments
(0.25 pmol) were mixed with the purified proteins and
incubated at 25°C for 2 h to perform binding reactions.
The binding reaction components were added following
the protocol as described in the LightShift Chemilumi-
nescent EMSA Kit (Thermo Scientific, Rockford, USA). The
binding reaction samples were run on a 6% polyacry-
lamide gel in 0.5× Tris-borate EDTA (TBE) and were
then transferred to nylon membranes. The membranes
were visualized using the Chemiluminescence Nucleic
Acid Detection Module Kit (Thermo Scientific).

DNase I footprinting assay

The FAM-labelled probe was generated by amplifying
the promoter region of pfiT using the 5’-end FAM-la-
belled forward primer (pfiT-FAM-f) and the reverse pri-
mer (pfiT-promoter-r; Table S1). For each reaction,
200 ng of FAM-labelled probes was mixed with a series
of amounts of PfiAT protein complex, and the mixtures
were incubated for 30 min at 25°C. Then, a series of
concentrations of DNase I (NEB, M0303S) was added to
cleave the DNA probes. A series of different incubation
time points was employed to achieve the best cutting
efficiency. The reaction was stopped by adding 200 mM
EDTA. Finally, the DNA was cleaned using the QIAEX II
Gel Extraction Kit (Qiagen, Hilden, Germany). The data
were obtained and analysed as described before (Wang
et al., 2012).

Phage production and plaque assay

Strains were grown overnight and adjusted to an OD₆₀₀
of 0.05 in 4 ml of LB in a 6-well plate. Pf4 phages were

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collected over time. In brief, two-millilitre culture from planktonic or pellicle PAO1 and ΔpflT strains was centrifuged at 12 000 rpm for 2 min. Then, the supernatants were filtered with 0.22 μm filters (Millipore Corporation, Billerica, MA, USA) to obtain pure PH4 phase solutions. The top-layer agar method was used to obtain bacterial lawns as previously described (Eisenstark, 1967).

**Quantitative reverse transcription real-time PCR (qRT-PCR)**

Strains grown for phage production were collected by centrifugation (12 000 rpm for 1 min) after keeping static for 6 h. The collected cell pellets were used for RNA extraction using an RNA extraction kit (Tiangen, Beijing, China). cDNA synthesis was conducted using reverse transcription kit with supplied random primers (Promega, Madison, WI, USA). The reverse transcription reaction mixes were incubated with procedures: room temperature incubation for 10 min, 42°C for 15 min, 95°C for 5 min and on ice for 5 min. Total cDNA (50 ng) was used for qRT-PCR using the Step One Real-Time PCR System. The level of the 16S rRNA gene transcript was used to normalize the gene expression data. The amplification efficiency of each primer set used was tested (Fig. S1), and they were comparable. Fold changes in the concentration of the targets were calculated as follows: $2^{\Delta \Delta C_{\text{t}}}$ = $2^{{\Delta C_{\text{t}}} - \Delta \Delta C_{\text{t}}}$, where $\Delta C_{\text{t}} = C_{\text{target}} - C_{\text{16S rRNA}}$ and $\Delta \Delta C_{\text{t}} = C_{\text{target}} - C_{\text{16S rRNA}}$. The ratio between CcdA and CcdB modulates the transcriptional repression of the ccd poison-antidote system. *Mol Microbiol* **41**: 73–82.

**References**

Afif, H., Allali, N., Couturier, M., and Van Melderen, L. (2001) The ratio between CcdA and CcdB modulates the
recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* **212**: 77–86.

Hoang, T.T., Kutchma, A.J., Becher, A., and Schweizer, H.P. (2000) Integration-proficient plasmids for *Pseudomonas aeruginosa*: Site-specific integration and use for engineering of reporter and expression strains. *Plasmid* **43**: 59–72.

Ilyina, T.S. (2015) Filamentous bacteriophages and their role in the virulence and evolution of pathogenic bacteria. *Mol Genet Microbiol Virol* **30**: 1–9.

Jiang, Y., Pogliano, J., Helinski, D.R., and Konieczny, I. (2002) ParE toxin encoded by the broad-host-range plasmid RK2 is an inhibitor of *Escherichia coli* gyrase. *Mol Microbiol* **44**: 971–979.

Karimova, G., Pidoux, J., Ullmann, A., and Ladant, D. (1998) A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc Natl Acad Sci USA* **95**: 5752–5756.

Kohanski, M.A., Dwyer, D.J., Hayete, B., Lawrence, C.A., and Collins, J.J. (2007) A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* **130**: 797–810.

Kroll, J., Klintner, S., Schneider, C., Voss, I., and Steinbuchel, A. (2010) Plasmid addiction systems: perspectives and applications in biotechnology. *Microb Biotechnol* **3**: 634–657.

Lehnerr, H., Maguin, E., Jafari, S., and Yarmolinsky, M.B. (1993) Plasmid addiction genes of bacteriophage-P1-Doc, which causes cell-death on curing of phage, and Phd, which prevents host death when phage is retained. *J Mol Biol* **233**: 414–428.

Li, M., Long, Y., Liu, Y., Liu, Y., Chen, R., Shi, J., et al. (2016) HigB of *Pseudomonas aeruginosa* enhances killing of phagocytes by up-regulating the type III secretion system in ciprofloxacin induced persister cells. *Front Cell Infect Microbiol* **6**: 125.

Li, Y., Liu, X., Tang, K., Wang, P., Zeng, Z., Guo, Y., and Wang, X. (2019) Excisionase in P1 filamentous prophage controls lysis-lysogeny decision-making in *Pseudomonas aeruginosa*. *Mol Microbiol* **111**: 495–513.

Liu, X., Li, Y., Guo, Y., Zeng, Z., Li, B., Wood, T.K., et al. (2015) Physiological function of Rac prophage during biofilm formation and regulation of Rac excision in *Escherichia coli* K-12. *Sci Rep* **5**: 16074.

Lyczak, J.B., Cannon, C.L., and Pier, G.B. (2002) Lung infections associated with cystic fibrosis. *Clin Microbiol Rev* **15**: 194–222.

Magnuson, R., and Yarmolinsky, M.B. (1998) Corepression of the P1 addiction operon by Phd and Doc. *J Bacteriol* **180**: 6342–6351.

Masuda, H., Tan, Q., Awano, N., Wu, K., and Inouye, M. (2012) YeeU enhances the bundling of cytoskeletal polymers of MreB and FtsZ, antagonizing the CbtA (YeeV) toxicity in *Escherichia coli*. *Mol Microbiol* **84**: 979–989.

Miller, J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Mrik, I., and Kobayashi, I. (2014) To be or not to be: regulation of restriction-modification systems and other toxin-antitoxin systems. *Nucleic Acids Res* **42**: 70–86.

Ogura, T., and Hiraga, S. (1983) Mini-F plasmid genes that couple host cell division to plasmid proliferation. *Proc Natl Acad Sci USA* **80**: 4784–4788.

Overgaard, M., Borch, J., Jorgensen, M.G., and Gerdes, K. (2008) Messenger RNA interferase RelE controls relBE transcription by conditional cooperativity. *Mol Microbiol* **69**: 841–857.

Palleroni, N.J. (1984) Pseudomonadaceae. In *Bergey’s Manual of Systematic Bacteriology*. Kreig, N.R., and Holt, J.G. (eds). Baltimore, MD: Williams & Wilkins, pp. 141–199.

Pecota, D.C., and Wood, T.K. (1996) Exclusion of T4 phage by the hok/sok killer locus from plasmid R1. *J Bacteriol* **178**: 2044.

Pesci, E.C., Pearson, J.P., Seed, P.C., and Iglewski, B.H. (1997) Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* **179**: 3127–3132.

Qiu, D., Damron, F.H., Mima, T., Schweizer, H.P., and Yu, H.D. (2008) PBAD-based shuttle vectors for functional analysis of toxic and highly regulated genes in *Pseudomonas* and *Burkholderia* spp. and other bacteria. *Appl Environ Microbiol* **74**: 7422–7426.

Rice, S.A., Tan, C.H., Mikkelsen, P.J., Kung, V., Woo, J., Tay, M., et al. (2009) The biofilm life cycle and virulence of *Pseudomonas aeruginosa* are dependent on a filamentous prophage. *ISME J* **3**: 271–282.

Roberts, R.C., Strom, A.R., and Helinski, D.R. (1994) The parDE operon of the broad-host-range plasmid RK2 specifies growth-inhibition associated with plasmid loss. *J Mol Biol* **237**: 35–51.

Secor, P.R., Sweere, J.M., Michaels, L.A., Malkovskiy, A.V., Lazzareschi, D., Katznelson, E., et al. (2015) Filamentous bacteriophage promote biofilm assembly and function. *Cell Host Microbe* **18**: 549–559.

Shanks, R.M.Q., Calazza, N.C., Hinsa, S.M., Toutain, C.M., and O’Toole, G.A. (2006) Saccharomyces cerevisiae-based molecular tool kit for manipulation of genes from gram-negative bacteria. *Appl Environ Microbiol* **72**: 5027–5036.

Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J., et al. (2000) Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* **406**: 959–964.

Sweere, J.M., Van Belleghem, J.D., Ishak, H., Bach, M.S., Popescu, M., Sunkari, V., et al. (2019) Bacteriophage trigger antiviral immunity and prevent clearance of bacterial infection. *Science* **363**: eaat9691.

Tumbull, K.J., and Gerdes, K. (2017) HicA toxin of *Escherichia coli* derepresses hicAB transcription to selectively produce HicB antitoxin. *Mol Microbiol* **104**: 781–792.

Wang, X., and Wood, T.K. (2011) Toxin-antitoxin systems influence biofilm and persister cell formation and the general stress response. *Appl Environ Microbiol* **77**: 5577–5583.

Wang, X.X., and Wood, T.K. (2016) Cryptic prophages as targets for drug development. *Drug Resist Update* **27**: 30–38.

Wang, X.X., Kim, Y., Ma, Q., Hong, S.H., Pokusaeva, K., Sturino, J.M., and Wood, T.K. (2010) Cryptic prophages help bacteria cope with adverse environments. *Nat Commu* **1**: 147.
Characterization of a new GlnR binding box in the promoter of amtB in Streptomyces coelicolor inferred a PhoP/GlnR competitive binding mechanism for transcriptional regulation of amtB. *J Bacteriol* **194**: 5237–5244.

Williams, J.J., Halvorsen, E.M., Dwyer, E.M., DiFazio, R.M., and Hergenrother, P.J. (2011) Toxin-antitoxin (TA) systems are prevalent and transcribed in clinical isolates of *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus*. *FEMS Microbiol Lett* **322**: 41–50.

Winther, K.S., and Gerdes, K. (2012) Regulation of enteric vapBC transcription: induction by VapC toxin dimer-breaking. *Nucleic Acids Res* **40**: 4347–4357.

Wood, T.L., and Wood, T.K. (2016) The HigB/HigA toxin/antitoxin system of *Pseudomonas aeruginosa* influences the virulence factors pyochelin, pyocyanin, and biofilm formation. *Microbiologyopen* **5**: 499–511.

Yao, J.Y., Guo, Y.X., Wang, P.X., Zeng, Z.S., Li, B.Y., Tang, K.H., *et al.* (2018) Type II toxin/antitoxin system ParEso/CopAso stabilizes prophage CP4So in *Shewanella oneidensis*. *Environ Microbiol* **20**: 1224–1239.

Zeng, Z., Liu, X., Yao, J., Guo, Y., Li, B., Li, Y., *et al.* (2016) Cold adaptation regulated by cryptic prophage excision in *Shewanella oneidensis*. *ISME J* **10**: 2787–2800.

Zhang, Y.Y., Xia, B., Li, M., Shi, J., Long, Y.Q., Jin, Y.X., *et al.* (2018) HigB reciprocally controls biofilm formation and the expression of type iii secretion system genes through influencing the intracellular c-di-gmp level in *Pseudomonas aeruginosa*. *Toxins* **10**(11): 424.

**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Primers used in this study.

**Fig. S1.** Real-time PCR standard curves and amplification efficiencies of primers in Fig. 3B. The genomic DNA of PAO1 was 10-fold serial diluted and RT-PCR was performed for gene amplification. The threshold cycle (CT) of each concentration was used as Y-axis and the log of input DNA was used as X-axis, and the real-time PCR standard curves were calculated, and the amplify efficiencies were calculated based on the following formula: 

$$E = (10^{-\frac{1}{slope_-1}}) \times 100.$$