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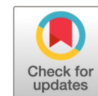


# The Two-Component System FleS/FleR Represses H1-T6SS via Cyclic di-GMP Signaling in *Pseudomonas aeruginosa*

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**ABSTRACT** The type VI secretion system (T6SS) is an important translocation apparatus that is widely employed by Gram-negative bacteria to deliver toxic effectors into eukaryotic and prokaryotic target cells, causing host damage and providing competitive advantages in polymicrobial environments. The genome of *Pseudomonas aeruginosa* harbors three T6SS clusters (H1-T6SS, H2-T6SS, H3-T6SS). Activities of these systems are tightly regulated by a complicated signaling network which remains largely elusive. In this study, we focused on a previously characterized two-component system FleS/FleR, and performed comparative transcriptome analysis between the PAO1 wild-type strain and its isogenic Δ*fleR* mutant, which revealed the important role of FleS/FleR in regulating multiple physiological pathways including T6SS. Gene expression and bacterial killing assays showed that the expression and activity of H1-T6SS are repressed in the wild-type strain owing to the high intracellular c-di-GMP content. Further explorations demonstrated that c-di-GMP relies on the transcription factor FleQ to repress H1-T6SS and its synthesis is controlled by a global regulator AmrZ which is induced by the active FleS/FleR. Interestingly, repression of H1-T6SS by FleS/FleR in PAO1 is independent of RetS which is known to regulate H1-T6SS by controlling the central post-transcriptional factor RsmA. Together, our results identified a novel regulator of H1-T6SS and provided detailed mechanisms of this signaling pathway in PAO1.

**IMPORTANCE** *Pseudomonas aeruginosa* is an opportunistic human pathogen distributed widely in the environment. The genome of this pathogen contains three T6SS clusters which contribute significantly to its virulence. Understanding the complex regulatory network that controls the activity of T6SS is essential for the development of effective therapeutic treatments for *P. aeruginosa* infections. In this study, transcriptome analysis led to the identification of a novel regulator FleS/FleR which inversely regulates H1-T6SS and H2-T6SS in *P. aeruginosa* PAO1. We further revealed a detailed FleS/FleR-mediated regulatory pathway of H1-T6SS in PAO1 which involves two additional transcriptional regulators AmrZ and FleQ and the second messenger c-di-GMP, providing important implications to develop novel anti-infective strategies and antimicrobial drugs.

**KEYWORDS** *Pseudomonas aeruginosa*, two-component system, FleS/FleR, T6SS, c-di-GMP

The type VI secretion system (T6SS), first discovered in 2006, is an important virulence determinant distributed in more than 200 Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Burkholderia thailandensis*, *Vibrio cholerae*, *Serratia marcescens,* and so forth (1–5). This system is composed of a set of core conserved genes including TssA-TssM, Hcp, VgrG, and ClpV to form its key structure which shares high similarity with the puncturing device of tailed bacteriophages (6). T6SS is

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known as a versatile secretion system. In addition to its function as a contractile molecular syringe to deliver toxins into neighboring competitors or translocate protein effectors into host cells which results in growth inhibition or death of the target cells, T6SS is involved in multiple other physiological processes such as biofilm formation and metal acquisition (7–9).

*P. aeruginosa* is an important opportunistic Gram-negative pathogen which can cause a variety of acute and chronic infections. Three independent T6SS clusters, namely, H1-T6SS, H2-T6SS, and H3-T6SS, have been identified in the genome of *P. aeruginosa* so far. H1-T6SS is the first discovered and well-characterized T6SS machinery displaying antibacterial activity by delivering at least seven different protein effectors, e.g., Tse1 to Tse7, providing a fitness advantage for *P. aeruginosa* in competition with other bacteria and contributing significantly to its infections in hosts (10–12). For example, Tse1 exhibits strong peptidoglycan-degrading activity which endows *P. aeruginosa* with a great ability to lyse bacterial competitors (13). Different from the H1-T6SS, the H2-T6SS and H3-T6SS can target both prokaryotic and eukaryotic cells using the effectors such as PldA and PldB (14–16).

T6SS activity in *P. aeruginosa* is tightly regulated at (post)transcriptional and (post) translational levels. Accumulating evidence has shown that T6SS is regulated by many factors such as the quorum sensing system (QS), copper-responsive regulator CueR, RNase YbeY, global regulator AmrZ, endogenous membrane stress, and the threonine phosphorylation pathway (TPP) (7, 17–22). Despite these progresses, the dominant factor controlling T6SS activity in *P. aeruginosa* is still regarded as the RNA binding protein RsmA which represses T6SS at the post-transcriptional level (22). RsmA is located downstream of the Gac/Rsm cascade which is responsible for the transitions of acute and chronic phases of *P. aeruginosa* infections (23). In this cascade, GacS phosphorylates GacA leading to the production of two small RNAs *rsmY* and *rsmZ* which are capable of sequestering RsmA and consequently de-repress the translation of T6SS mRNAs (24). Activity of the Gac/Rsm pathway is inversely controlled by two additional histidine kinases LadS and RetS which stimulates and represses the activity of the signaling pathway, respectively (25, 26). During *in vitro* growth, it has been reported that T6SS is poorly assembled in the strains with ordinary expression of *retS* and deletion of *retS* is required to obtain a fully active T6SS in *P. aeruginosa* (12, 27). However, whether additional regulatory factors exist to modulate T6SS activity remains largely unknown.

Two-component systems (TCSs) represent a group of important bacterial regulatory factors that control expression profiles of many genes or pathways in response to changing environments (28). In *P. aeruginosa*, a TCS FleS/FleR was previously characterized to mainly regulate bacterial motility and biofilm formation (29, 30). While interestingly, unlike canonical TCSs which consist of a transmembrane sensor histidine kinase, the histidine kinase FleS was found not containing a transmembrane domain (29). In order to comprehensively understand the biological roles of this peculiar TCS, in this study, we performed transcriptome analysis and discovered that FleS/FleR is a novel regulator of T6SS. We further investigated the detailed molecular mechanisms of how FleS/FleR controlled H1-T6SS activity in PAO1 and showed a RetS-independent signaling cascade that was composed of AmrZ, c-di-GMP, and FleQ. These findings expanded our understanding in the complexity of T6SS regulation and provided significant implications for therapeutic treatments for *P. aeruginosa* infections.

## RESULTS

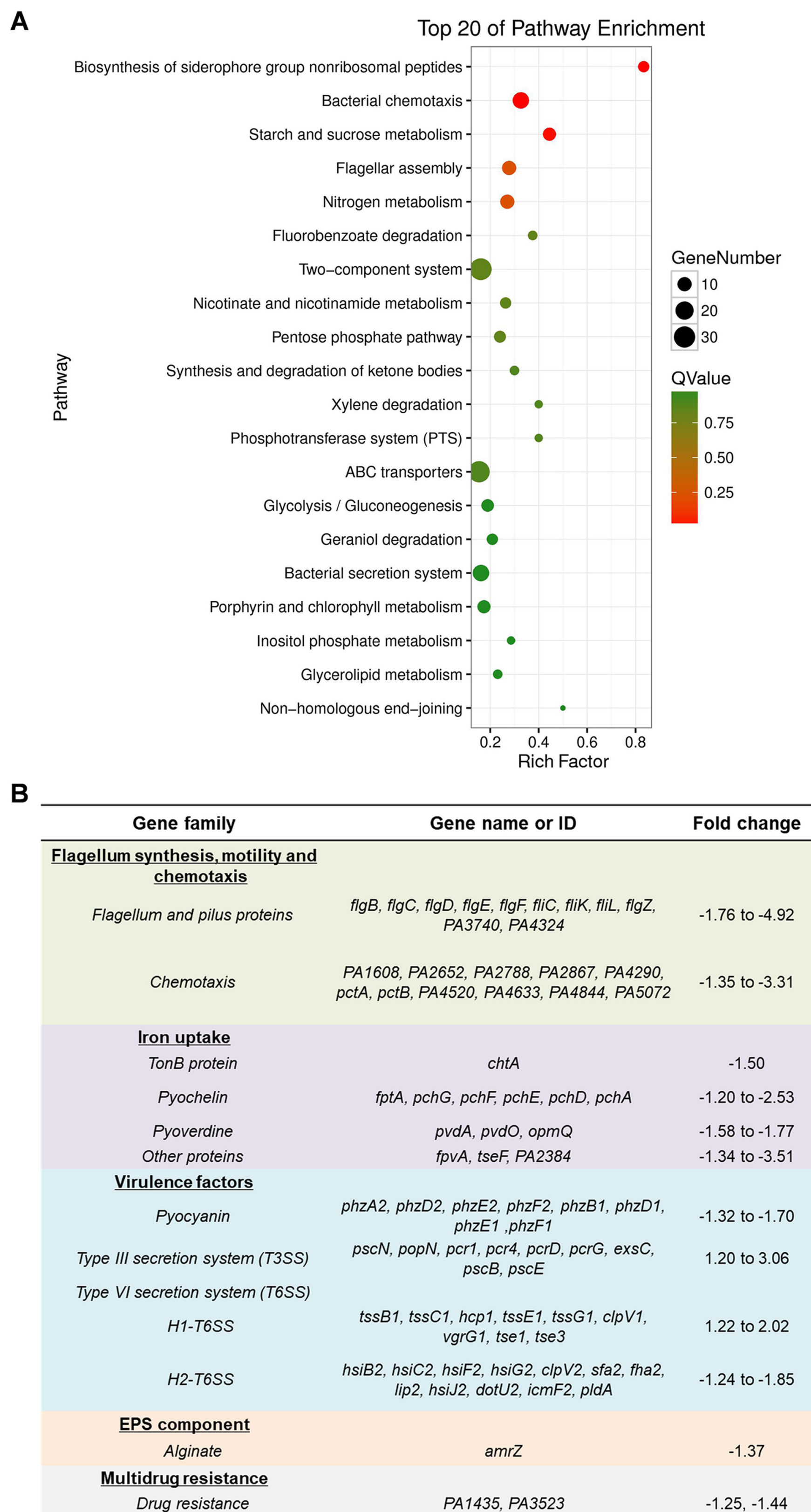
**FleS/FleR regulates multiple physiological pathways in PAO1.** The TCS FleS/FleR plays important roles in regulating biofilm formation and motility in *P. aeruginosa* (29, 30). To further investigate whether this TCS is involved in regulating other physiological pathways in *P. aeruginosa*, we deleted *fleR* in PAO1 and conducted RNA-sequencing (RNA-seq) to compare its transcriptomic profiles with the wild-type PAO1 strain (PAO1 WT). A total of 440 differentially expressed genes with more than 1.2-log2fold changes were identified between these two strains (Table S1). Specifically, 121 genes were downregulated and

319 genes were upregulated in the *fleR* deletion mutant. To summarize the differentially expressed genes into interpretable pathways, enrichment analysis was performed for these genes based on the KEGG pathways (31), which showed that these genes were enriched in 20 pathways such as bacterial chemotaxis, flagellar assembly, two-component system, ABC transporters, and bacterial secretion system, etc. (Fig. 1A). As listed in Fig. 1B, a number of these genes were found to be associated with bacterial virulence and antibiotic resistance. Some of them were related to flagellar synthesis, motility, and chemotaxis, and this was in accordance with the previously reported regulatory functions of FleS/FleR in biofilm formation and motility (29).

**FleR differentially regulates the expression of three T6SS clusters in PAO1 and repressed H1-T6SS is the major reason causing reduced antibacterial capacity.** PAO1 contains three T6SS gene clusters in its genome which are named as H1-T6SS, H2-T6SS, and H3-T6SS, respectively (Fig. 2A) (5). It was interesting to notice that the expression of H1-T6SS and H2-T6SS were inversely regulated in the absence of *fleR* and expression changes of H3-T6SS genes were not detected (Fig. 1B), suggesting that FleS/FleR could be a novel regulator of T6SS in *P. aeruginosa* which displayed different regulatory mechanisms on different T6SS clusters. To verify the RNA-seq result, we selected four genes from each T6SS locus and performed RT-qPCR to examine their expression. Consistent with the RNA-seq result, deletion of *fleR* in PAO1 resulted in significantly upregulated expression of H1-T6SS genes, downregulated expression of H2-T6SS genes and undetectable expression changes of H3-T6SS genes (Fig. 2B to D), which indicated that FleR negatively regulated H1-T6SS and positively regulated H2-T6SS. Given that H1-T6SS in PAO1 is the first discovered and the most well-studied system (10, 12), in the present study, we decided to focus on H1-T6SS and aimed to reveal the molecular mechanisms underlying the regulation of FleS/FleR on this system.

To further confirm whether H1-T6SS is repressed by FleR, we selected a structural gene *hcp1* and an effector-encoding gene *tse1* from H1-T6SS and constructed the chromosomal His-tagged *hcp1* and *tse1* in PAO1 WT and Δ*fleR* strains to examine their protein productions by conducting the Western blot assay. Consistent with the RT-qPCR result, it showed that productions of both Hcp1 and Tse1 proteins were substantially increased in the Δ*fleR* strain which could be completely abolished with the ectopic expression of FleR (Fig. 2E). Considering that H1-T6SS is essential for the antibacterial activity of *P. aeruginosa* and Tse1 plays an important role in cell lysis of the competing bacterial cells (10), we moved to investigate whether increased expression of the H1-T6SS in Δ*fleR* could enhance its anti-bacterial ability. We assessed the antibacterial abilities of PAO1 WT and Δ*fleR* by performing a bacterial killing assay using *E. coli* as the prey. The survival rate of *E. coli* was significantly reduced when it was cocultured with Δ*fleR* compared with the PAO1 WT (Fig. 2F). The survival rate of *E. coli* was recovered when it was cocultured with Δ*fleR* which was complemented with an FleR-expressing vector (Fig. 2F), demonstrating that FleR repressed the antibacterial activity of H1-T6SS. We also found that Tse1 was the key antibacterial effector regulated by FleR because further deletion of *tse1* in PAO1 Δ*fleR* completely abolished its killing ability (Fig. 2F). These results demonstrated that FleR inhibited the antibacterial activity of PAO1 by repressing the expression of H1-T6SS and its main effector Tse1.

**FleR regulates H1-T6SS in a c-di-GMP-dependent manner.** Interestingly, RNA-seq and RT-qPCR results showed that a group of diguanylate cyclase (DGCs) genes responsible for c-di-GMP synthesis including *siaD*, *gcbA*, *sadC*, *mucR*, and *PA4929* were significantly downregulated in the Δ*fleR* strain (Table S1 and Fig. S1A); however, phosphodiesterases (PDEs) genes such as *bifA*, *PA2071*, and *PA3825* responsible for c-di-GMP degradation were not influenced (Fig. S1B). Based on these observations in combination with a previous report that c-di-GMP inactivates T6SS activity in *Agrobacterium tumefaciens* (32), we speculated that FleR regulated H1-T6SS by modulating intracellular c-di-GMP contents. To examine this speculation, we first evaluated the intracellular c-di-GMP contents in PAO1 WT and Δ*fleR* strains using the expression of *cdrA* which is directly responsive to changing cellular c-di-GMP levels as an indicator (33–35). It was shown that deletion of *fleR* led to a significantly reduced expression of *cdrA* and such




| Gene family | Gene name or ID | Fold change |
|---|---|---|
| **Flagellum synthesis, motility and chemotaxis** | | |
| *Flagellum and pilus proteins* | *flgB, flgC, flgD, flgE, flgF, fliC, fliK, fliL, flgZ, PA3740, PA4324* | -1.76 to -4.92 |
| *Chemotaxis* | *PA1608, PA2652, PA2788, PA2867, PA4290, pctA, pctB, PA4520, PA4633, PA4844, PA5072* | -1.35 to -3.31 |
| **Iron uptake** | | |
| *TonB protein* | *chtA* | -1.50 |
| *Pyochelin* | *fptA, pchG, pchF, pchE, pchD, pchA* | -1.20 to -2.53 |
| *Pyoverdine* | *pvdA, pvdO, opmQ* | -1.58 to -1.77 |
| *Other proteins* | *fpvA, tseF, PA2384* | -1.34 to -3.51 |
| **Virulence factors** | | |
| *Pyocyanin* | *phzA2, phzD2, phzE2, phzF2, phzB1, phzD1, phzE1 ,phzF1* | -1.32 to -1.70 |
| *Type III secretion system (T3SS)* | *pscN, popN, pcr1, pcr4, pcrD, pcrG, exsC, pscB, pscE* | 1.20 to 3.06 |
| *Type VI secretion system (T6SS)* | | |
| *H1-T6SS* | *tssB1, tssC1, hcp1, tssE1, tssG1, clpV1, vgrG1, tse1, tse3* | 1.22 to 2.02 |
| *H2-T6SS* | *hsiB2, hsiC2, hsiF2, hsiG2, clpV2, sfa2, fha2, lip2, hsiJ2, dotU2, icmF2, pldA* | -1.24 to -1.85 |
| **EPS component** | | |
| *Alginate* | *amrZ* | -1.37 |
| **Multidrug resistance** | | |
| *Drug resistance* | *PA1435, PA3523* | -1.25, -1.44 |

**FIG 1** FleS/FleR regulates multiple physiological pathways in PAO1. (A) KEGG-enrichment of differentially expressed genes identified in the RNA-seq analysis. The *y* axis represents the names of the pathways. The *x* axis represents the rich factor which refers to the ratio of the number of differentially expressed genes to the total number of genes in the pathway. The size of the dot represents the number of differentially

(Continued on next page)

reduction could be fully restored by ectopic expression of FleR (Fig. S1C), indicating that FleR acted as an activator of c-di-GMP synthesis and higher intracellular c-di-GMP content potentially inhibited the H1-T6SS activity. We next examined the regulatory role of c-di-GMP in Δ*fleR* by either deleting or ectopically expressing a DGC gene *sadC*. Further deletion of *sadC* in Δ*fleR* did not cause obvious changes of *hcp1* and *tse1* expression (Fig. 3A and B, Fig. S2A), which was reasonable because *sadC* was already repressed to a low expression level in the absence of *fleR*. Interestingly, ectopic expression of *sadC* in Δ*fleR* to increase the c-di-GMP content completely abolished the elevated expression of *hcp1* and *tse1* (Fig. 3C and D, Fig. S2B). Consistently, the enhanced antibacterial activity of Δ*fleR* was abolished with the ectopic expression of *sadC* (Fig. 3E). In contrast, ectopic expression of a PDE gene *bifA* in Δ*fleR* did not show any differences on the *hcp1* and *tse1* expression as well as the antibacterial activity of this mutant (Fig. 3C to E, Fig. S2B). These results suggested that c-di-GMP mediated the regulation of FleR on H1-T6SS in PAO1.

Given that ectopic expression of the native DGC and PDE genes might coordinate other cellular activities other than to solely increase the c-di-GMP content as we expected, we chose two more genes (a DGC gene *W909_14945* and a PDE gene *W909_14950*) involved in c-di-GMP metabolism from *Dickeya zeae* EC1 (36) and expressed them in Δ*fleR*, respectively. Consistent with the native genes *sadC* and *bifA*, expression of the heterologous DGC gene *W909_14945* totally abolished the elevated expression of *hcp1* and *tse1* as well as the antibacterial activity of Δ*fleR* while *W909_14950* did not generate any differences (Fig. 3C to E, Fig. S2B). These results confirmed that FleR functions to activate c-di-GMP synthesis which consequently inhibits the H1-T6SS activity.

**FleQ represses H1-T6SS activity in response to c-di-GMP.** It is known that c-di-GMP exerts its regulatory roles through interacting with effector proteins or RNAs (37). Reported effectors include proteins containing the PilZ domain, degenerate GGDEF or EAL domains, transcriptional regulators, and mRNA riboswitches (38–40). In *P. aeruginosa*, proteins such as FleQ and FlgZ have been identified as c-di-GMP effectors to regulate a variety of physiological processes including bacterial chemotaxis, biofilm formation, motility, etc. (38, 39, 41, 42). We therefore moved to investigate whether the activity of H1-T6SS regulated by c-di-GMP was mediated by the effectors FleQ or FlgZ. We deleted FleQ and FlgZ in the Δ*fleR* mutant, respectively, and then introduced plasmids to ectopically express the DGC gene *sadC*. RT-qPCR showed that ectopic expression of *sadC* to increase intracellular c-di-GMP was incapable of restoring the *hcp1* and *tse1* expression in Δ*fleR* to the wild-type level when FleQ was absent (Fig. 4A, Fig. S3). Western blot examination and bacterial killing assay further confirmed that FleQ was indispensable to repress H1-T6SS activity under the c-di-GMP overproducing condition (Fig. 4B and C). In contrast, further deletion of *flgZ* in Δ*fleR* had no effect on the reduced *tse1* expression and antibacterial activity during ectopic expression of *sadC* (Fig. 4A to C). Moreover, in the PAO1 WT which possessed an active FleR and contained relatively high c-di-GMP level, deletion of FleQ also increased the *hcp1* and *tse1* expression as well as the antibacterial activity (Fig. 4A to C, Fig. S3). These results demonstrated that FleQ was the key effector that responded to c-di-GMP and played an essential role in mediating the regulation of H1-T6SS activity by FleR.

It has been demonstrated that FleQ regulates expression of flagellar and biofilm genes by directly binding to the conserved sequence in the promoters of genes such as *flhA*, *filE*, *fliL*, *psl*, *pel*, etc. (41, 43). Therefore, we searched potential FleQ binding sites in promoters of H1-T6SS genes by aligning the promoter sequences with the consensus DNA binding sequence for FleQ. Sequence alignment identified two putative FleQ binding sites in the

**FIG 1** Legend (Continued)
expressed genes in the pathways, and the color of the dot represents different Q values. The higher the value of rich factor represents the greater the enrichment degree. The smaller the Q value represents the more significant the enrichment. (B) Selected gene families related to bacterial virulence and drug resistance with more than 1.2-log2fold changes owing to the deletion of *fleR* in PAO1 (Δ*fleR* versus PAO1 WT).

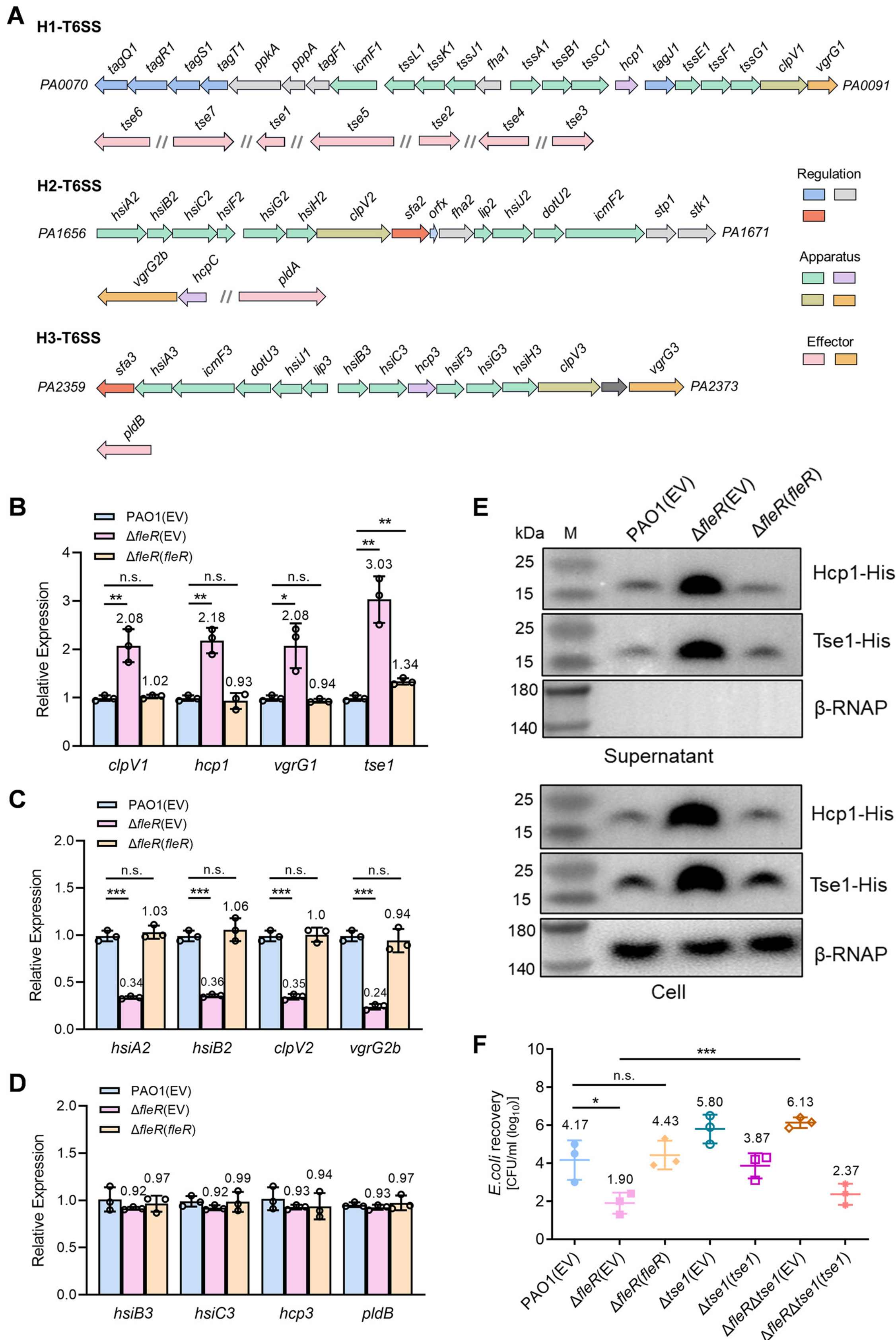



**FIG 2** FleR differentially regulates the expression of three T6SS clusters in PAO1 and repressed H1-T6SS is the major reason causing reduced anti-bacterial capacity of PAO1. (A) A diagram showing the three T6SS gene clusters in *P. aeruginosa* PAO1. (B)

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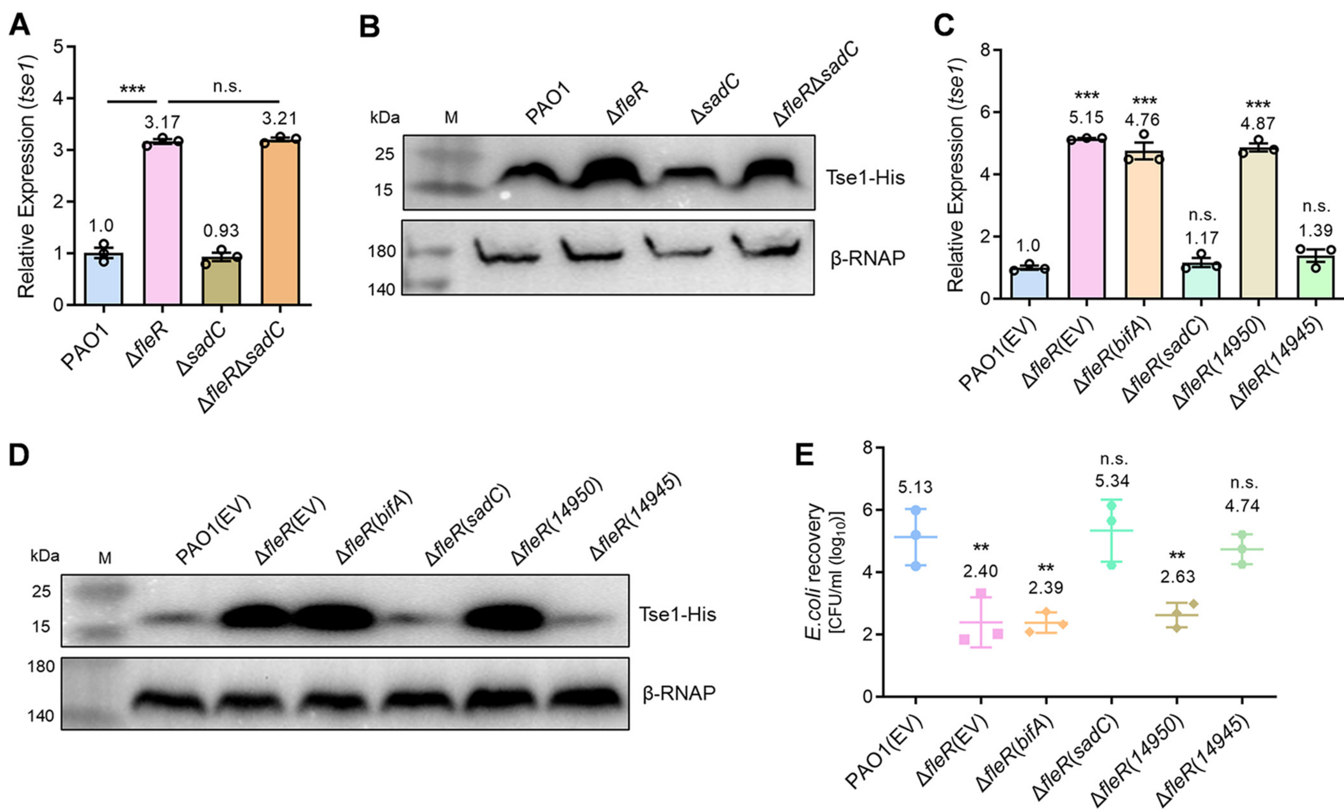


**FIG 3** FleR regulates H1-T6SS in a c-di-GMP-dependent manner. (A) Relative expression of *tse1* measured by RT-qPCR in the PAO1, Δ*fleR*, Δ*sadC*, and Δ*fleR* Δ*sadC* strains. (B) Western blot analysis of Tse1-His in the cell-associated protein fractions from PAO1, Δ*fleR*, Δ*sadC*, and Δ*fleR* Δ*sadC* strains. (C) Relative expression of *tse1* measured by RT-qPCR in the strains of PAO1, Δ*fleR*, and Δ*fleR* with ectopic expression of DGCs and PDEs. (D) Western blot analysis of Tse1-His in the cell-associated protein fractions from the PAO1, Δ*fleR*, and Δ*fleR* strains with ectopic expression of DGCs and PDEs. (E) Bacterial killing assay between the indicated PAO1 strains and the *E. coli* prey. Data are represented as the mean ± SD ($n$ = 3). **, $P < 0.01$; ***, $P < 0.001$ *versus* the indicated group based on paired Student's $t$ test (A) or *versus* PAO1(EV) based on one-way ANOVA (C, E). n.s., not significant; EV, empty vector for the control.

promoter regions of the H1-T6SS structural gene cluster *tssA1B1C1* and the effector gene *tse1*, respectively, which shared high similarities with the FleQ DNA binding consensus sequence (Fig. 4D). This analysis suggested that FleQ might regulate H1-T6SS activity by directly interacting with the T6SS promoters.

**The regulation of c-di-GMP synthesis and H1-T6SS activity by FleR is mediated by AmrZ.** To understand how FleR induced intracellular c-di-GMP contents, we next purified FleR protein and conducted electrophoretic mobility shift assay (EMSA) to examine whether FleR could bind to the promoters of DGC genes to trigger their transcription. However, none of the five DGC genes promoters *siaD*, *gcbA*, *sadC*, *mucR*, or *PA4929* showed interaction with FleR (Fig. S4), implying that FleR did not induce the expression of these DGC genes directly. Interestingly, our RNA-seq data in combination with RT-qPCR verification identified a global regulator gene *amrZ* which was significantly downregulated in the Δ*fleR* mutant (Fig. 1B and 5A). AmrZ is conserved in pseudomonads and has been reported as a major determinant of intracellular c-di-GMP levels by controlling the expression of a complex network of DGC and PDE genes in *Pseudomonas fluorescens* F113 and regulates bacterial motility in a c-di-GMP-dependent manner in *P. aeruginosa*

**FIG 2** Legend (Continued)
Relative expression of H1-T6SS genes *clpV1*, *hcp1*, *vgrG1*, and *tse1* measured by RT-qPCR in the strains of PAO1, Δ*fleR* and Δ*fleR* with ectopic expression of *fleR* (Δ*fleR*(*fleR*)). (C) Relative expression of H2-T6SS genes *hsiA2*, *hsiB2*, *clpV2*, and *vgrG2b* measured by RT-qPCR in PAO1, Δ*fleR*, and Δ*fleR*(*fleR*) strains. (D) Relative expression of H3-T6SS genes *hsiB3*, *hsiC3*, *hcp3*, and *pldB* measured by RT-qPCR in PAO1, Δ*fleR*, and Δ*fleR*(*fleR*) strains. (E) Western blot analysis of Hcp1-His and Tse1-His in the cell-associated and concentrated supernatant protein fractions from PAO1, Δ*fleR*, and Δ*fleR*(*fleR*) strains. The RNA polymerase $\beta$ subunit ($\beta$-RNAP) was selected as an internal control for the assay. (F) Bacterial killing assay between the PAO1 strains and the *E. coli* prey. Data are represented as the mean ± SD ($n$ = 3). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *versus* the indicated group based on paired Student's $t$ test. n.s., not significant; EV, empty vector for the control.

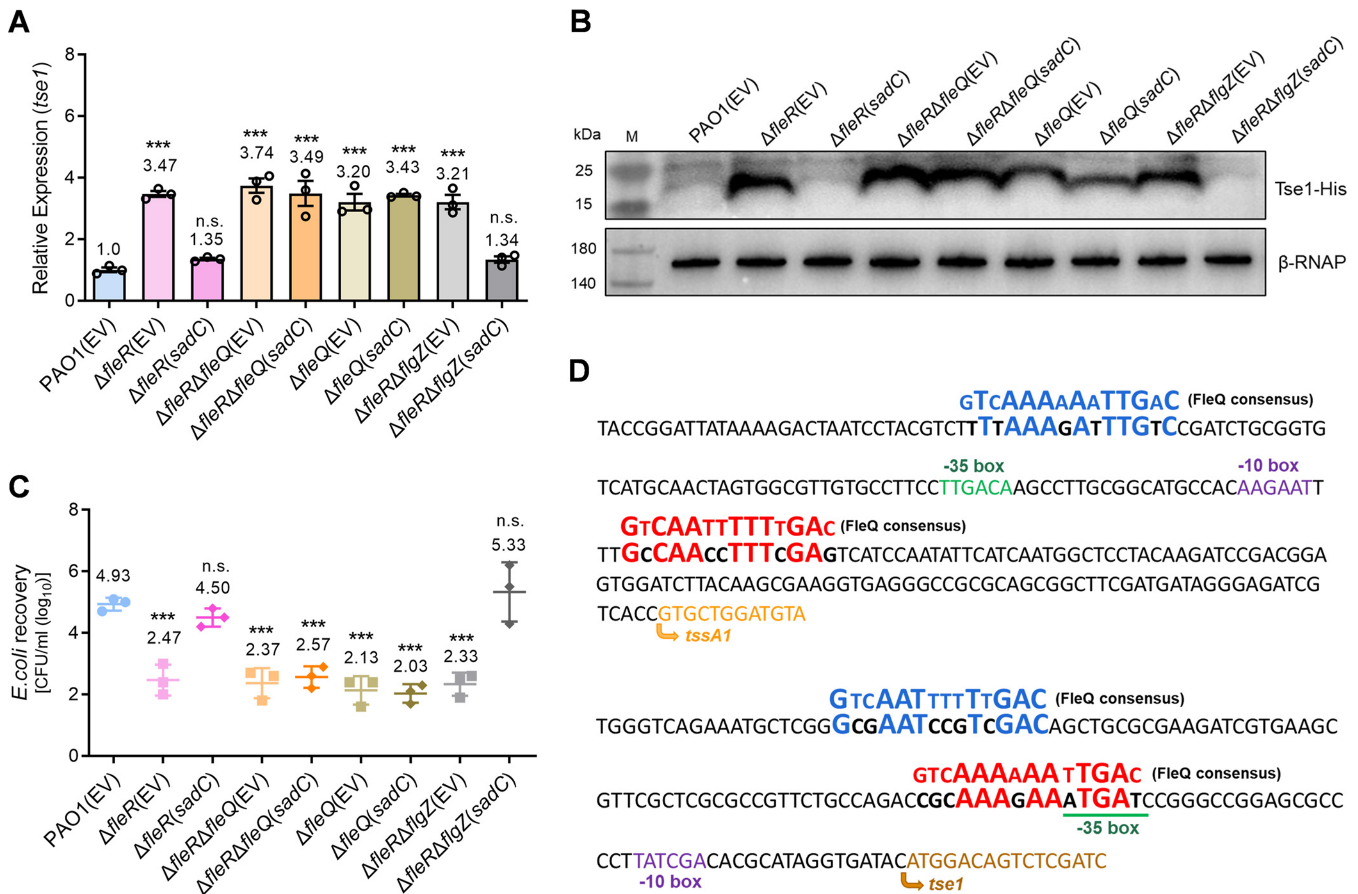


**FIG 4** FleQ is the effector that responds to intracellular c-di-GMP and modulates the activity of H1-T6SS. (A) Relative expression of *tse1* measured by RT-qPCR in the indicated PAO1 strains. (B) Western blot analysis of Tse1-His in the cell-associated protein fractions from the indicated PAO1 strains. (C) Bacterial killing assay between the indicated PAO1 strains and the *E. coli* prey. (D) Prediction of FleQ binding sites in the promoters of the gene cluster *tssA1B1C1* and the effector gene *tse1* by searching for the FleQ consensus sites (in blue and in red). Data are represented as the mean ± SD ($n = 3$). ***, $P < 0.001$ *versus* PAO1(EV) based on one-way ANOVA. n.s., not significant. EV, empty vector for the control.

PA14 (44, 45). Therefore, we supposed that AmrZ could be the primary target of FleR which subsequently modulated the c-di-GMP content and H1-T6SS activity in PAO1. To test this hypothesis, we expressed *amrZ* in the Δ*fleR* mutant and monitored the *hcp1* and *tse1* expression. RT-qPCR showed that ectopic expression of *amrZ* significantly reduced the expression of both genes in Δ*fleR* to the level similar as that in the PAO1 WT while ectopic expression of *amrZ* in PAO1 WT did not show obvious expression changes compared with the vector control (Fig. 5B, Fig. S5). Moreover, Western blot analysis and bacterial killing assay displayed that ectopic expression of *amrZ* in Δ*fleR* significantly reduced its Tse1 production and antibacterial activity, respectively (Fig. 5C and D), confirming that AmrZ mediated the regulation of H1-T6SS.

We next assessed the intracellular c-di-GMP content in Δ*fleR* with the ectopic expression of *amrZ*, which showed a significant increase of the c-di-GMP content in this strain relative to the Δ*fleR* strain containing the vector control (Fig. 5E). Moreover, we deleted *fleQ* in the Δ*fleR* strain with the ectopic expression of *amrZ* and examined the expression of *hcp1* and *tse1* as well as the antibacterial activity to further ensure the role of *amrZ* in H1-T6SS regulation was mediated by c-di-GMP and FleQ. As shown in Fig. 5B to D and Fig. S5, deletion of FleQ completely blocked the AmrZ-mediated repression of H1-T6SS. These results demonstrated that AmrZ transduced the signal from FleR to increase intracellular c-di-GMP synthesis and thereafter promoted H1-T6SS activity.

**FleR regulates H1-T6SS in a novel pathway which is independent of RetS.** RetS is an important sensor kinase that represses H1-T6SS in *P. aeruginosa* via the Gac/Rsm pathway where RsmA is suggested as a central regulator silencing the translation of T6SS mRNAs (26).

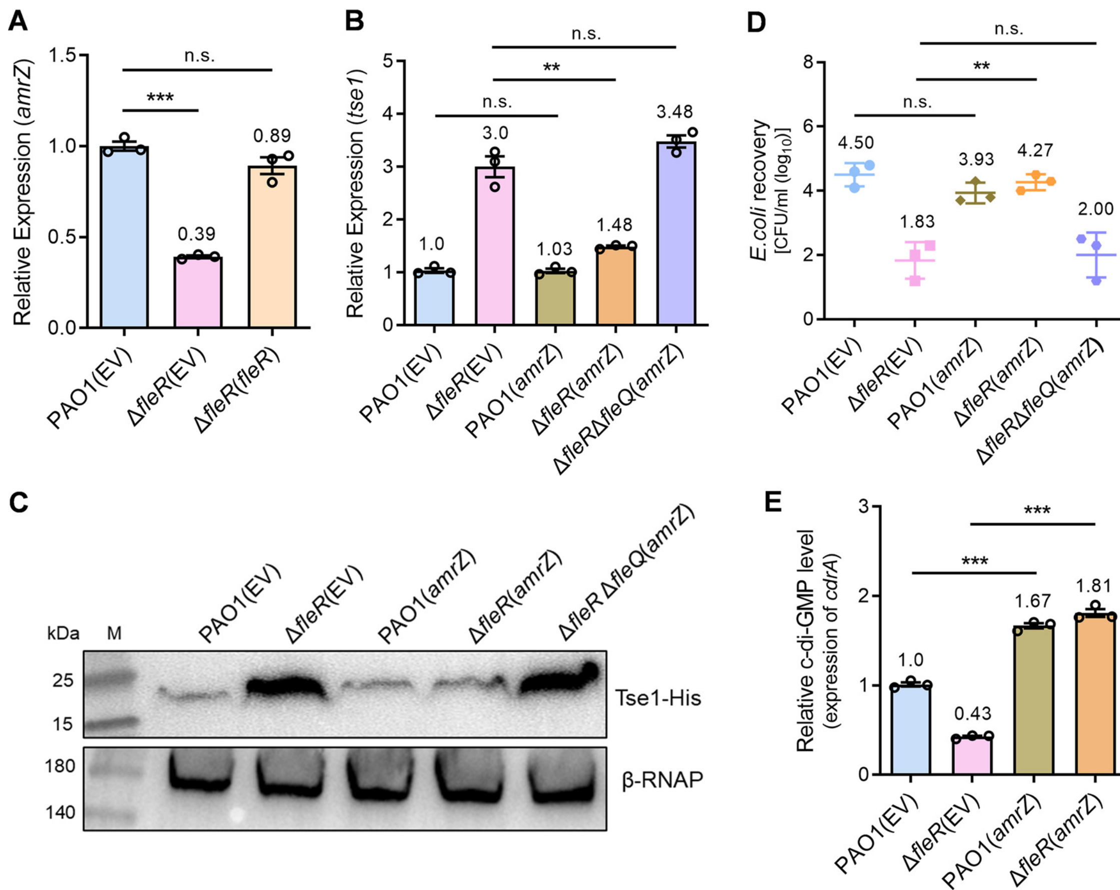


**FIG 5** The regulation of c-di-GMP synthesis and H1-T6SS activity by FleR is mediated by AmrZ. (A) Relative expression of *amrZ* measured by RT-qPCR in PAO1, Δ*fleR*, and Δ*fleR*(*fleR*) strains. (B) Relative expression of *tse1* measured by RT-qPCR in the strains of PAO1 and Δ*fleR* with ectopic expression of *amrZ*. (C) Western blot analysis of Tse1-His in the cell-associated protein fractions from the strains of PAO1 and Δ*fleR* with ectopic expression of *amrZ*. (D) Bacterial killing assay between the indicated PAO1 strains and the *E. coli* prey. (E) Relative intracellular c-di-GMP levels in the indicated PAO1 strains were determined by measuring the relative expression of *cdrA*. Data are represented as the mean ± SD ($n = 3$). **, $P < 0.01$; ***, $P < 0.001$ *versus* the indicated group based on paired Student's *t* test. n.s., not significant; EV, empty vector for the control.

It has been well demonstrated that mutation of RetS is necessary to achieve a fully active H1-T6SS in *P. aeruginosa* under laboratory conditions (46). Moreover, it was reported that RetS switches expression of T3SS and H1-T6SS in a c-di-GMP-dependent manner in the *P. aeruginosa* PAK strain (47). Here, whether FleR interplayed with RetS in regulating H1-T6SS was not known. To clarify this, we further constructed a *retS* deletion mutant Δ*retS* and a *fleR retS* double deletion mutant Δ*fleR* Δ*retS*, and measured *hcp1* and *tse1* expression and antibacterial activity of these mutants. Δ*fleR* and Δ*retS* single deletion mutants displayed similar levels of the *hcp1* and *tse1* expression as well as the antibacterial activity, which were significantly higher than the PAO1 WT (Fig. 6A to C, Fig. S6). Intriguingly, double deletion of *fleR* and *retS* resulted in an additive effect of two single deletion mutants on the *hcp1* and *tse1* expression and the antibacterial activity (Fig. 6A to C, Fig. S6), suggesting that FleR and RetS pathways were independent in regulating H1-T6SS. We also determined FleR expression in Δ*retS* and RetS expression in Δ*fleR*, which showed no differences in both cases (Fig. 6D), further indicating that these two pathways were independent.

## DISCUSSION

The T6SS is a powerful weapon employed by many Gram-negative bacteria to subvert or kill neighboring prey cells via injecting toxins and protein effectors (48). Understanding the regulation of T6SS in pathogens is important owing to the great contributions of T6SS in causing host infectious diseases and maintaining competitive advantages in polymicrobial

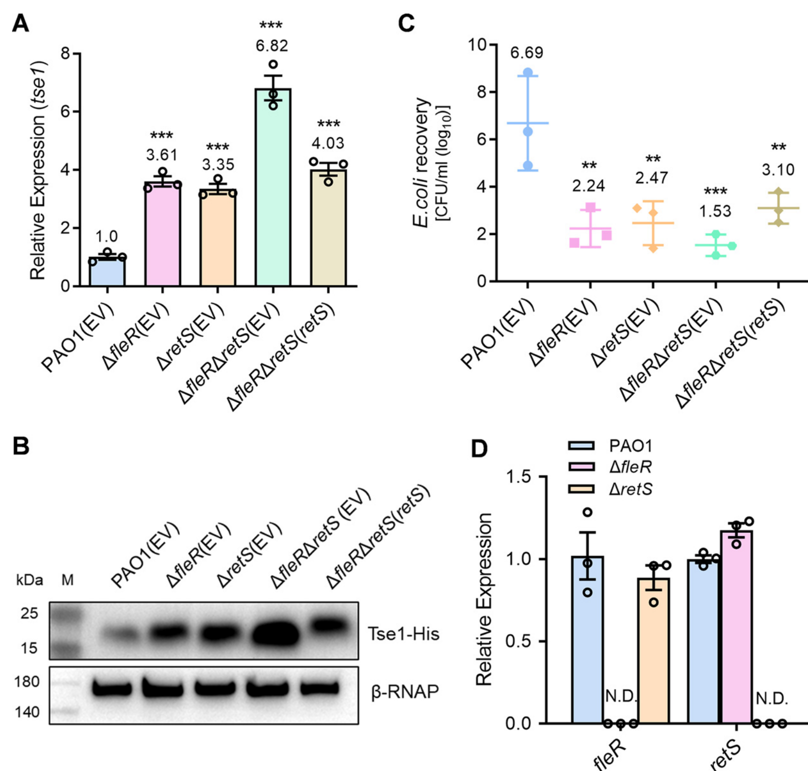


**FIG 6** FleR and RetS repress the activity of H1-T6SS independently. (A) Relative expression of *tse1* measured by RT-qPCR in the PAO1, Δ*fleR*, Δ*retS*, and Δ*fleR* Δ*retS* strains. (B) Western blot analysis of Tse1-His in the cell-associated protein fractions from the strains of PAO1, Δ*fleR*, Δ*retS*, and Δ*fleR* Δ*retS*. (C) Bacterial killing assay between the indicated PAO1 strains and the *E. coli* prey. (D) Relative expression of *fleR* and *retS* in the PAO1, Δ*fleR*, and Δ*retS* strains. Data are represented as the mean ± SD ($n = 3$). **, $P < 0.01$; ***, $P < 0.001$ *versus* PAO1(EV) based on one-way ANOVA. N.D., not detected; EV, empty vector for the control.

communities (12). *P. aeruginosa* is a notorious opportunistic human pathogen equipped with three T6SS gene clusters and represents a huge threat of lifelong infections. Despite increasing efforts that have been made to elucidate the structure, biogenesis, and secreted effectors of T6SS in recent years, regulation of T6SS activity is still largely unclear. Facilitated by high-throughput RNA-seq analysis, we discovered the two-component system FleS/FleR is a novel regulator of T6SS. Interestingly, FleS/FleR regulates three T6SS clusters in PAO1 in completely different manners, e.g., downregulation of H1-T6SS, upregulation of H2-T6SS and no changed expression of H3-T6SS. In this study, we focused on the elucidation of the regulatory mechanism of H1-T6SS and demonstrated that FleR can induce the intracellular c-di-GMP content in an AmrZ-dependent manner and the elevated c-di-GMP further represses the H1-T6SS expression and antibacterial activity of PAO1 through the transcription regulator FleQ. Moreover, this regulatory circuit showed independency of the RetS pathway (Fig. 7).

FleS/FleR was previously identified to control biofilm formation and motility in *P. aeruginosa* (49). In this study, our RNA-seq result further revealed that FleS/FleR are also involved in regulating many other physiological pathways, including those associated with bacterial virulence and antibiotic resistance, highlighting the versatile regulatory roles of FleS/FleR in host adaptation. Unfortunately, physiologically relevant signals of FleS/FleR have not yet been identified, so it is still unclear how FleS/FleR is activated or repressed in responsive to the host environment. Because deletion of *fleR* significantly downregulated virulence traits related to acute infections such as motility, chemotaxis, iron uptake, and pyocyanin production, it seems that FleS/FleR is probably activated at the early stage of infection and functions predominantly to establish acute infections. Although we found that FleS/FleR represses the activity of H1-T6SS, which is a typic response during acute infection, it is still confusing how H2-T6SS is induced by FleS/FleR and what is the physiological function of the induced H2-T6SS.

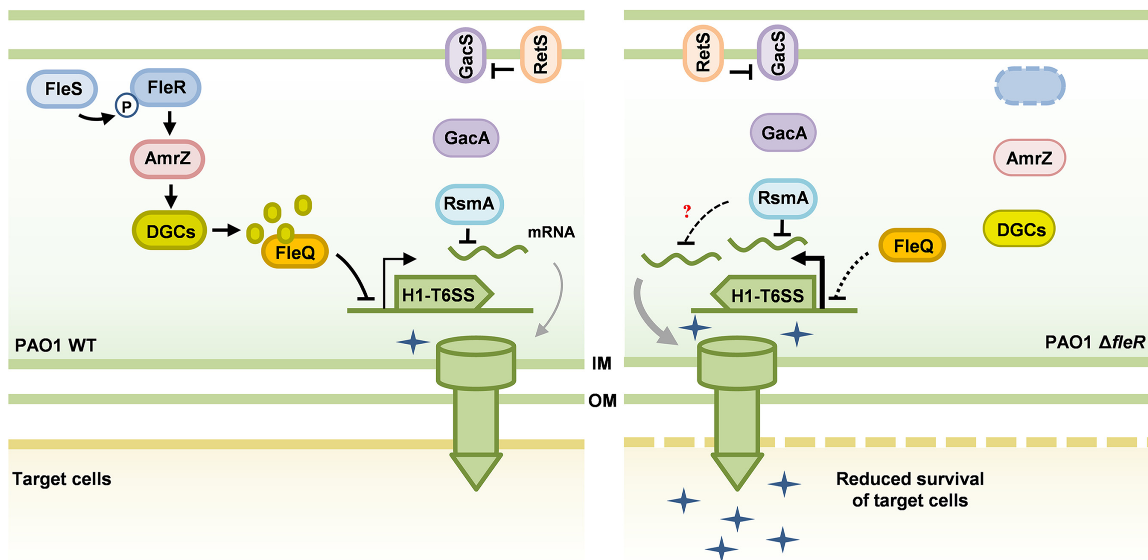


**FIG 7** A model illustrating the regulation of H1-T6SS expression by FleS/FleR in PAO1. H1-T6SS is poorly expressed in PAO1 WT under laboratory conditions, which is mainly achieved by the RetS/Gac/Rsm pathway. The RetS/Gac/Rsm pathway regulates H1-T6SS at post-transcriptional level by release or sequester the central factor RsmA. In this study, we revealed a novel pathway under the control of the two-component system FleS/FleR which is independent of the RetS/Gac/Rsm pathway. FleS/FleR activates AmrZ to induce the synthesis of intracellular c-di-GMP, which subsequently represses the expression and activity of the H1-T6SS through FleQ. In the Δ*fleR* mutant, reduced c-di-GMP content leads to the de-repression and activation of H1-T6SS regardless of the presence of RetS and RsmA with unknown mechanisms.

AmrZ is a conserved global regulator in pseudomonads which binds hundreds of genomic regions to control many physiological pathways implicated in environmental adaptation (50, 51). A previous study demonstrated that AmrZ represses H2-T6SS and activates H1-T6SS and H3-T6SS through directly binding to T6SS promoters in PA14 (22). However, in PAO1, we showed that AmrZ inactivates H1-T6SS under the regulation of FleR, indicating the different regulatory patterns among *P. aeruginosa* isolates. Moreover, the regulation of H1-T6SS in this case seems more possibly dependent on the intracellular c-di-GMP contents rather than directly controlled by AmrZ. This is supported by the strong negative correlation between the H1-T6SS activity and the intracellular c-di-GMP content and the strong positive correlation between the intracellular c-di-GMP content and the AmrZ expression level we observed. We examined the expression of five major DGC genes to look for the potential target of AmrZ. Although we found three genes *siaD*, *sadC*, and *PA4929*, were upregulated significantly in Δ*fleR* with the ectopic expression of *amrZ*, all only showed a slight fold change (~2 fold) (Fig. S7). A comprehensive evaluation of all the DGCs and PDEs is required to determine the role of AmrZ in regulating intracellular c-di-GMP contents. On the other hand, we showed that AmrZ is a key component bridging FleS/FleR and H1-T6SS, but how AmrZ is controlled by FleR requires further investigation. In this study, EMSA examination excluded the possibility of direct binding of FleR to the AmrZ promoter (Fig. S4) and RNA-seq analysis did not show significant expression changes of sigma factor genes such as *algU*, which was known to control the AmrZ expression (data not shown) (52).

Although T6SS expression is regulated by a complicated signaling network in *P. aeruginosa*, RsmA is regarded as the dominant regulator repressing T6SS activity by blocking the translation of all the T6SS mRNAs. Therefore, de-repression of T6SS by sequestering RsmA is a prerequisite for investigations on T6SS activity, which is commonly achieved by the deletion of *retS*. Interestingly, we found that deletion of FleR is adequate to induce the activity of H1-T6SS at both transcriptional and translational levels in PAO1 as evidenced by the RT-qPCR and Western blot examinations. This induction is independent of RetS, and simultaneous deletion of *fleR* and *retS* led to an additive induction of the H1-T6SS activity. However, it is still unknown how the signaling bypasses the RetS/Gac/Rsm pathway in PAO1

because it has been shown that all T6SS mRNAs are silenced by RsmA in spite of the regulation of T6SS transcripts by AmrZ in PA14 (22). Nonetheless, these results further highlight the complexity of the regulation of T6SS and the discrepancies of signaling pathways among *P. aeruginosa* isolates (53). Notably, H1-T6SS and H2-T6SS components were detected at 25°C in PA14 even in the presence of RetS and RsmA (22), which indicated that expression of H1-T6SS does not require the sequestration of RsmA under some peculiar conditions. Thus, it will be interesting to figure out whether additional factors exist to receive signals from FleS/FleR and regulate H1-T6SS activity in PAO1.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, primers, and culture conditions.** Strains and plasmids used in this study are summarized in Table 1. Primers used in this study are listed in Table 2. The plasmid pK18mobsacB was used for gene deletion in PAO1. The plasmids pBBR1-MCS5 and pUCP18 (for bacterial killing assays) were used for gene complementation. Unless indicated otherwise, PAO1 and its isogenic mutants were cultured at 37°C in Luria-Bertani (LB) broth (Tryptone 10g/L, Yeast extract 5g/L, NaCl 10g/L). Antibiotics were added in the medium when necessary: carbenicillin, 400 $\mu$g/mL for *P. aeruginosa* and 100 $\mu$g/mL for *E. coli*; gentamicin, 50 $\mu$g/mL for *P. aeruginosa* and *E. coli*; kanamycin, 50 $\mu$g/mL for *E. coli*; ampicillin, 100 $\mu$g/mL for *E. coli*.

**Construction of PAO1 mutants and gene complementation assay.** A SacB-based strategy was employed to achieve gene deletion in PAO1 according to a previous description (54). Briefly, 500-bp upstream and 500-bp downstream sequences of the target gene were amplified by PCR with *Pfu* DNA polymerase (Vazyme, China). The PCR products were assembled into the suicide plasmid pK18mobsacB which was predigested with BamHI and HindIII. The constructed plasmid pK18-*fleR* was introduced into PAO1 using the helper plasmid pRK2013 by triparental mating. Desired mutants were counter-selected using LB plates containing 10% sucrose and verified by PCR and DNA sequencing. For gene complementation, the open reading frames (ORF) together with its native promoter was amplified by PCR using primers listed in Table 2. PCR fragments were ligated into the downstream of the *lac* promoter of pBBR1-MCS5 or pUCP18 between the HindIII and BamHI sites or BamHI and HindIII sites, respectively. The plasmids were verified by PCR and DNA sequencing and introduced into PAO1 strains by tri-parental mating. Successful plasmid delivery into the PAO1 strains was confirmed by PCR.

**RT-qPCR.** Bacterial cells were grown in LB medium and harvested at $OD_{600}$ of 1.5 by centrifugation. Total RNA was isolated using the RNeasy minikit (Qiagen, Germany) according to the manufacturer's instructions. The cDNA samples were synthesized from the isolated total RNA using SuperScript II reverse transcriptase (Invitrogen, USA) and random primers (Invitrogen, USA). qPCR was performed using the QuantiTect SYBR green PCR kit (Qiagen, Germany) on the ABI QuantStudioTM6 Flex system (Roche, Switzerland) according to the manufacturer's instructions. The 50S ribosomal protein gene *rplU* was selected as an internal control (55). The relative gene expression level was calculated by using the $2^{-\Delta\Delta CT}$ method and presented as the mean of three independent biological isolates.

**RNA-seq.** The enriched mRNA was fragmented as 200 nt to 700 nt and reverse transcribed into cDNA with random primers. Second-strand cDNA was synthesized by DNA polymerase I, RNase H, and dNTP. The cDNA fragments were then purified with QiaQuick PCR extraction kit with end repaired and poly(A) added and ligated to Illumina sequencing adapters. The ligation products were size selected by agarose gel electrophoresis, followed by PCR amplification, and sequencing by Illumina HiSeq TM 2500 (Gene Denovo Biotechnology Co., China). Differentially expressed genes with ≥1.2-log2fold changes were identified at a false discovery rate (FDR) of ≤0.05, and analyzed using the major public pathway-related database KEGG (56). The formula for calculating the *P* value is

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

where *N* is the number of all genes that with annotation in database, *n* is the number of differentially expressed genes in *N*, *M* is the number of all genes annotated to specific pathways, and *m* is the number of differentially expressed genes in *M*. The calculated *P* value has gone through FDR correction, taking FDR of ≤0.05 as a threshold. Q value is the *P* value underwent multiple hypothesis test corrections. The value ranges from 0 to 1 with more significant when it is closer to 0.

**Protein purification and Western blot analysis.** Overnight bacterial cultures were 1:1,000 diluted into 10-mL fresh LB medium and incubated until $OD_{600}$ reached 1.5. Bacterial cultures were chilled on ice for 20 min. For each sample, bacterial pellet was collected by centrifugation and lysed by radio immunoprecipitation assay (RIPA) lysis buffer (Biosharp, China). The supernatant was purified with a 0.22-$\mu$m filter and treated with trichloroacetic acid (TCA) at a final concentration of 10%. The precipitate was pelleted by centrifugation and resuspended in SDS loading buffer after washing with ice-cold acetone for three times. Western blot analysis was performed according to the method described previously (57). Proteins were separated by SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane by electroblotting. The PVDF membrane was blocked with 5% (wt/vol) skim milk in PBST buffer (PBS supplemental with 0.05% Tween 20) for 1 h, followed by immunoblotting using anti-His antibody (Abbkine, USA) and horseradish peroxidase-conjugated goat anti-mouse antibody (Abbkine, USA).

**TABLE 1** Bacterial strains and plasmids used in this study

| Strains or plasmids | Relevant genotype or phenotype | Source or reference |
|---|---|---|
| *P. aeruginosa* strains | | |
| PAO1 | Wild-type strain | Lab collection |
| Δ*fleR* | PAO1 with the deletion of the *fleR* gene | This study |
| Δ*fleR*(EV) | Δ*fleR* containing the empty plasmids pBBR1-MCS5 or pUCP18 | This study |
| Δ*fleR*(*fleR*) | Δ*fleR* containing the expression constructs pBBR1-MCS5-*fleR* or pUCP18-*fleR* | This study |
| Δ*fleR*(*sadC*) | Δ*fleR* containing the expression constructs pBBR1-MCS5-*sadC* or pUCP18-*sadC* | This study |
| Δ*fleR*(*bifA*) | Δ*fleR* containing the expression constructs pBBR1-MCS5-*bifA* or pUCP18-*bifA* | This study |
| Δ*fleR*(*14945*) | Δ*fleR* containing the expression constructs pBBR1-MCS5-*W909_14945* or pUCP18-*W909_14945* | This study |
| Δ*fleR*(*14950*) | Δ*fleR* containing the expression constructs pBBR1-MCS5-*W909_14950* or pUCP18-*W909_14950* | This study |
| Δ*fleQ* | PAO1 with the deletion of the *fleQ* gene | This study |
| Δ*fleQ*(EV) | Δ*fleQ* containing the empty plasmids pBBR1-MCS5 or pUCP18 | This study |
| Δ*fleQ*(*sadC*) | Δ*fleQ* containing the expression construct pBBR1-MCS5-*sadC* | This study |
| Δ*fleR*Δ*fleQ* | PAO1 with the deletion of the *fleR* and *fleQ* genes | This study |
| Δ*fleR*Δ*fleQ*(EV) | Δ*fleR*Δ*fleQ* containing the empty plasmids pBBR1-MCS5 or pUCP18 | This study |
| Δ*fleR*Δ*fleQ*(*sadC*) | Δ*fleR*Δ*fleQ* containing the expression constructs pBBR1-MCS5-*sadC* or pUCP18-*sadC* | This study |
| Δ*tse1* | PAO1 with the deletion of the *tse1* gene | This study |
| Δ*tse1*(EV) | Δ*tse1* containing the empty plasmids pBBR1-MCS5 or pUCP18 | This study |
| Δ*tse1*(*tse1*) | Δ*tse1* containing the expression construct pUCP18-*tse1* | This study |
| Δ*fleR*Δ*tse1* | PAO1 with the deletion of the *fleR* and *tse1* genes | This study |
| Δ*fleR*Δ*tse1*(*tse1*) | Δ*fleR*Δ*tse1* containing the expression construct pUCP18-*tse1* | This study |
| Δ*sadC* | PAO1 with the deletion of the *sadC* gene | This study |
| Δ*fleR*Δ*sadC* | PAO1 with the deletion of the *fleR* and *sadC* genes | This study |
| Δ*fleR*Δ*flgZ* | PAO1 with the deletion of the *fleR* and *flgZ* genes | This study |
| Δ*fleR*Δ*flgZ*(*sadC*) | Δ*fleR*Δ*flgZ* containing the expression constructs pBBR1-MCS5-*sadC* or pUCP18-*sadC* | This study |
| PAO1(*amrZ*) | PAO1 containing the expression constructs pBBR1-MCS5-*amrZ* or pUCP18-*amrZ* | This study |
| Δ*fleR*(*amrZ*) | Δ*fleR* containing the expression constructs pBBR1-MCS5-*amrZ* or pUCP18-*amrZ* | This study |
| Δ*fleR*Δ*fleQ*(*amrZ*) | Δ*fleR*Δ*fleQ* containing the expression constructs pBBR1-MCS5-*amrZ* or pUCP18-*amrZ* | This study |
| Δ*retS* | PAO1 with the deletion of the *retS* gene | This study |
| Δ*retS*(EV) | Δ*retS* containing the empty plasmids pBBR1-MCS5 or pUCP18 | This study |
| Δ*fleR*Δ*retS* | PAO1 with the deletion of the *fleR* and *retS* genes | This study |
| Δ*fleR*Δ*retS*(EV) | Δ*fleR*Δ*retS* containing the empty plasmids pBBR1-MCS5 or pUCP18 | This study |
| Δ*fleR*Δ*retS*(*retS*) | Δ*fleR*Δ*retS* containing the expression constructs pBBR1-MCS5-*retS* or pUCP18-*retS* | This study |
| *E. coli* strains | | |
| DH5α | *spuE44 ΔlacU169(φ80lacZΔM15) hsdR17λpir recA1 endA1 gyrA96 thi-1 relA1* | Lab collection |
| pRK2013 | $Tra^+$, Mob-, ColE1-replicon, $Kan^r$, $Spe^r$ | Lab collection |
| Plasmids | | |
| pBBR1-MCS5 | Broad-host-range cloning vector; $Gm^r$ | Lab collection |
| pUCP18 | *E. coli-P. aeruginosa* shuttle expression vector with $P_{lac}$, $Amp^r$, $Car^r$ | Lab collection |
| pBBR1-MCS5-*fleR* | pBBR1-MCS5 containing *fleR* under the control of $P_{lac}$ | This study |
| pUCP18-*fleR* | pUCP18 containing *fleR* under the control of $P_{lac}$ | This study |
| pBBR1-MCS5-*sadC* | pBBR1-MCS5 containing *sadC* under the control of $P_{lac}$ | This study |
| pUCP18-*sadC* | pUCP18 containing *sadC* under the control of $P_{lac}$ | This study |
| pBBR1-MCS5-*bifA* | pBBR1-MCS5 containing *bifA* under the control of $P_{lac}$ | This study |
| pUCP18-*bifA* | pUCP18 containing *bifA* under the control of $P_{lac}$ | This study |
| pUCP18-*tse1* | pUCP18 containing *tse1* under the control of $P_{lac}$ | This study |
| pBBR1-MCS5-*W909_14945* | pBBR1-MCS5 containing *W909_14945* under the control of $P_{lac}$ | This study |
| pUCP18-*W909_14945* | pUCP18 containing *W909_14945* under the control of $P_{lac}$ | This study |
| pBBR1-MCS5-*W909_14950* | pBBR1-MCS5 containing *W909_14950* under the control of $P_{lac}$ | This study |
| pUCP18-*W909_14950* | pUCP18 containing *W909_14950* under the control of $P_{lac}$ | This study |
| pBBR1-MCS5-*retS* | pBBR1-MCS5 containing *retS* under the control of $P_{lac}$ | This study |
| pUCP18-*retS* | pUCP18 containing *retS* under the control of $P_{lac}$ | This study |
| pBBR1-MCS5-*amrZ* | pBBR1-MCS5 containing *amrZ* under the control of $P_{lac}$ | This study |
| pUCP18-*amrZ* | pUCP18 containing *amrZ* under the control of $P_{lac}$ | This study |
| pK18mobsacB | Broad-host-range sucrose counter-selection allelic exchange vector, *sacB*, $Gm^r$ | Lab collection |
| pK18-*fleR* | pK18 containing *fleR* flanking regions for generation of *fleR* in-frame deletion | This study |
| pK18-*tse1* | pK18 containing *tse1* flanking regions for generation of *tse1* in-frame deletion | This study |
| pK18-*retS* | pK18 containing *retS* flanking regions for generation of *retS* in-frame deletion | This study |
| pK18-*fleQ* | pK18 containing *fleQ* flanking regions for generation of *fleQ* in-frame deletion | This study |
| pK18-*flgZ* | pK18 containing *flgZ* flanking regions for generation of *flgZ* in-frame deletion | This study |
| pK18-*sadC* | pK18 containing *sadC* flanking regions for generation of *sadC* in-frame deletion | This study |
| pK18-*tse1*-his | pK18 containing *tse1*. Six repeated CAC was added at the C-terminal. | This study |
| pK18-*hcp1*-his | pK18 containing *hcp1*. Six repeated CAC was added at the C-terminal. | This study |

**TABLE 2** PCR primers used in this study

| Primers | Sequence (5′ to 3′) | Description |
|---|---|---|
| For construction | | |
| *fleR*-Up-F | gagctcggtacccggggatccGCTGGTGTTCGCCCGCGG | For amplification of the 5′-region of *fleR* |
| *fleR*-Up-R | agcacggggttactcctgaaTCGCAG | |
| *fleR*-Dn-F | ttcaggagtaaccccgtgctCGCCATGTTCCCC | For amplification of the 3′-region of *fleR* |
| *fleR*-Dn-R | acgacggccagtgccaagcttACGCTGGCCTTCTGGCTG | |
| *fleR*-FC-F | gtcgacggtatcgataagcttTGCGGGCCCGAACTGCGC | For construction of pBBR1-MCS5-*fleR* |
| *fleR*-FC-R | cgctctagaactagtggatccGCGGACGCAAAAGGCCCG | |
| *fleR*-PC-F | gagctcggtacccggggatccTGCGGGCCCGAACTGCGC | For construction of pUCP18-*fleR* |
| *fleR*-PC-R | acgacggccagtgccaagcttGGACGCAAAAGGCCCGCA | |
| *tse1*-Up-F | agctcggtacccggggatccGACTGTACTTTCACCCAGCT | For amplification of the 5′-region of *tse1* |
| *tse1*-Up-R | acggcctgaagtatcacctaTGCGTGTCGATAAGG | |
| *tse1*-Dn-F | taggtgatacttcaggccgtGCTGCGAATG | For amplification of the 3′-region of *tse1* |
| *tse1*-Dn-R | cgacggccagtgccaagcttTCTCGATGGCCTGGATCACG | |
| *tse1*-PC-F | gagctcggtacccggggatccACGCATAGGTGATACATGGA | For construction of pUCP18-*tse1* |
| *tse1*-PC-R | acgacggccagtgccaagcttATTCGCAGCACGGCCTGAA | |
| *retS*-Up-F | gagctcggtacccggggatccCATGGTCCGCCTGGAGTCC | For amplification of the 5′-region of *retS* |
| *retS*-Up-R | ggcgaagtcccttcgaagg | |
| *retS*-Dn-F | ccttcgaagggacttcgccGGGCAGCGACGTGCTCCG | For amplification of the 3′-region of *retS* |
| *retS*-Dn-R | cgacggccagtgccaagcttATAGAGCACCAGCATCTTCA | |
| *retS*-FC-F | gtcgacggtatcgataagcttGGCACCGCGCTGAAGGAT | For construction of pBBR1-MCS5-*retS* |
| *retS*-FC-R | cgctctagaactagtggatccATCCGCCGTGGCGGAGGC | |
| *retS*-PC-F | gagctcggtacccggggatccGGCACCGCGCTGAAGGAT | For construction of pUCP18-*retS* |
| *retS*-PC-R | acgacggccagtgccaagcttATCCGCCGTGGCGGAGGC | |
| *fleQ*-Up-F | gagctcggtacccggggatccATCGGTGAGCTGGATCAGGTC | For amplification of the 5′-region of *fleQ* |
| *fleQ*-Up-R | atccgattcgcgccacatttTGATC | |
| *fleQ*-Dn-F | aaatgtggcgcgaatcggatGATTGACAGGTCGTT | For amplification of the 3′-region of *fleQ* |
| *fleQ*-Dn-R | acgacggccagtgccaagcttCCTCGCGCGGAGCGAAGC | |
| *flgZ*-Up-F | gagctcggtacccggggatccTATCGGCCACGCCAACCA | For amplification of the 5′-region of *flgZ* |
| *flgZ*-Up-R | aacagttcgtcgaacgggttGGGCACCTT | |
| *flgZ*-Dn-F | aacccgttcgacgaactgttCTGAGTTTCACAGG | For amplification of the 3′-region of *flgZ* |
| *flgZ*-Dn-R | acgacggccagtgccaagcttTGCTGGAACGCGCCCTGC | |
| *sadC*-Up-F | gagctcggtacccggggatccTCCTGCTCTGGCTGGCGC | For amplification of the 5′-region of *sadC* |
| *sadC*-Up-R | actggtgacctcccacgtgtCCTGGTGCGCCTG | |
| *sadC*-Dn-F | acacgtgggaggtcaccagtGCCT | For amplification of the 3′-region of *sadC* |
| *sadC*-Dn-R | acgacggccagtgccaagcttGGTCGCAGAACGCGCCGG | |
| *sadC*-FC-F | gtcgacggtatcgataagcttCGGGTCGGCCAGGATCGA | For construction of pBBR1-MCS5-*sadC* |
| *sadC*-FC-R | cgctctagaactagtggatccAGGTTGCTGCCGGCGGCG | |
| *sadC*-PC-F | gagctcggtacccggggatccCGGGTCGGCCAGGATCGA | For construction of pUCP18-*sadC* |
| *sadC*-PC-R | acgacggccagtgccaagcttAGGTTGCTGCCGGCGGCG | |
| *bifA*-FC-F | gtcgacggtatcgataagcttCGACGTCTGGGAACACGC | For construction of pBBR1-MCS5-*bifA* |
| *bifA*-FC-R | cgctctagaactagtggatccCTGGGCAGCGCGCTATTG | |
| *bifA*-PC-F | gagctcggtacccggggatccCGACGTCTGGGAACACGC | For construction of pUCP18-*bifA* |
| *bifA*-PC-R | acgacggccagtgccaagcttCTGGGCAGCGCGCTATTG | |
| *amrZ*-FC-F | gtcgacggtatcgataagcttGGAGACTGTGTCAGCCCGG | For construction of pBBR1-MCS5-*amrZ* |
| *amrZ*-FC-R | cgctctagaactagtggatccAGCCGGCGAATGCCGGCC | |
| *amrZ*-PC-F | gagctcggtacccggggatccGGAGACTGTGTCAGCCCGG | For construction of pUCP18-*amrZ* |
| *amrZ*-PC-R | acgacggccagtgccaagcttAGCCGGCGAATGCCGGCC | |
| *W909_14945*-FC-F | gtcgacggtatcgataagcttAAATATTAGCCAGGCTTATGTCTATAACG | For construction of pBBR1-MCS5-*W909_14945* |
| *W909_14945*-FC-R | cgctctagaactagtggatccTACGACAGGCCCGGATGG | |
| *W909_14945*-PC-F | gagctcggtacccggggatccAAATATTAGCCAGGCTTATGTC | For construction of pUCP18-*W909_14945* |
| W909_14945-PC-R | acgacggccagtgccaagcttTACGACAGGCCCGGATGG | |
| *W909_14950*-FC-F | gtcgacggtatcgataagcttTACACAGCGTGGTTATGGTATAAACA | For construction of pBBR1-MCS5-*W909_14950* |
| *W909_14950*-FC-R | cgctctagaactagtggatccCTCTAATCAAATAAGCAGTAGTGACTCAGG | |
| *W909_14950*-PC-F | gagctcggtacccggggatccTACACAGCGTGGTTATGGTATA | For construction of pUCP18-*W909_14950* |
| *W909_14950*-PC-R | acgacggccagtgccaagcttCTCTAATCAAATAAGCAGTAGTG | |
| *tse1*-his-Up-F | gagctcggtacccggggatccGGGCCGGAGCGCCCCTTA | For amplification of the 5′-region of *tse1*. Six repeated CAC was added at the C-terminal. |
| *tse1*-his-Up-R | agcacggcctgaatcagtggtggtggtggtggtgactgGCCCTGGGCAGGCT | |
| *tse1*-his-Dn-F | cagtcaccaccaccaccaccacTGATTCAGGCCGTGCTGC | For amplification of the 3′-region of *tse1*. Six repeated CAC was added at the C-terminal. |
| *tse1*-his-Dn-R | acgacggccagtgccaagcttCGATGGCCTGGATCACGTC | |
| *hcp1*-his-Up-F | agctcggtacccggggatccCGGGAGGAAAGATGGCTGTT | For amplification of the 5′-region of *hcp1*. Six repeated CAC was added at the C-terminal. |
| *hcp1*-his-Up-R | tcatcagtggtggtggtggtggtgggcCTGCACGTTCTGG | |

(Continued on next page)

**TABLE 2** (Continued)

| Primers | Sequence (5′ to 3′) | Description |
|---|---|---|
| *hcp1*-his-Dn-F | gcccaccaccaccaccaccactgatgaGCCGGCTGCCGGTCA | For amplification of the 3′-region of *hcp1*. Six repeated CAC was added at the C-terminal. |
| *hcp1*-his-Dn-R | acgacggccagtgccaagcttCACCGGCGTCAGGCGCCC | |
| Sequences in lower case indicate the homology arms for recombination. | | |
| For qPCR: | | |
| q-*clpV1*-F | GTCTACGGCAGCCTGATGTT | |
| q-*clpV1*-R | GACCTTGAGCTTGGCGAA | |
| q-*hcp1*-F | TCCAAGGACAAGACTCACGC | |
| q-*hcp1*-R | CTTGGTGAACGACAGGTCCT | |
| q-*vgrG1*-F | CCGCATCTTCCAGAACCAG | |
| q-*vgrG1*-R | TGCACGCAGTATTCCCACTC | |
| q-*tse1*-F | CACCCCGAACAAGGACAAC | |
| q-*tse1*-R | TTGGTCCAGCTCTGCTCCA | |
| q-*hsiA2*-F | AAGCTCTCCTCGCATTATCTGG | |
| q-*hsiA2*-R | TTGCCCAGCTCATTTTCCAG | |
| q-*hsiB2*-F | TGACGCTGAGCGTACCCAAT | |
| q-*hsiB2*-R | CATCGCGCAACTCCATCAG | |
| q-*clpV2*-F | CATTGATCCTGGCCCTGCTA | |
| q-*clpV2*-R | GAGAGCGCGAAATCCTTCAAC | |
| q-*vgrG2b*-F | CGCATCTACCATGAGCACCT | |
| q-*vgrG2b*-R | CGAAGCGGAAGTAGTAGACCAG | |
| q-*hsiB3*-F | TCAACTATTTCGCCAACGGC | |
| q-*hsiB3*-R | GTAGCTGGCCGTTCAGGTAG | |
| q-*hsiC3*-F | ACGAAGAGGAATACGGCACC | |
| q-*hsiC3*-R | ACGTTCGACAGCTTCTCCAG | |
| q-*hcp3*-F | CTCAACGAGTATTGCTGCGC | |
| q-*hcp3*-R | TTGGAGAGCACCACGTTGTT | |
| q-*pldB*-F | AGTTGTGGGAGCTACATGCC | |
| q-*pldB*-R | TGTCACGACATCCCAGAAGC | |
| q-*amrZ*-F | AACTCCTACCTACTCCAGCCGT | |
| q-*amrZ*-R | GAGTTCATGCTGCGGTGATG | |
| q-*cdrA*-F | CCAGTTCAACCCCAACGAGA | |
| q-*cdrA*-R | GTCGAAGCCCTTCCAGTTGA | |
| q-*fleR*-F | GCCTGATCCGTACACGCTAC | |
| q-*fleR*-R | GAACGGCTTGACCAGGTAGTC | |
| q-*retS*-F | TGATCCAGCAGCTCAACCTG | |
| q-*retS*-R | GCTGATCTTGGCCAGGAACT | |
| q-*siaD*-F | CAGGGAGGAGAACGAACGCT | |
| q-*siaD*-R | TATTCGCGTAGCTCGGACTCC | |
| q-*gcbA*-F | CCGAATTGGCCAAGGTGAT | |
| q-*gcbA*-R | ATCGGCTTGGTGAGGAAGTC | |
| q-*sadC*-F | CGAACTCACCGGTCTGTTCA | |
| q-*sadC*-R | CCGCTTGAAGTGATCGAGGT | |
| q-*mucR*-F | AGATCGACCGTGGCTTCATC | |
| q-*mucR*-R | GGCGACGATCTTCAGGTTCA | |
| q-*PA4929*-F | ATCTGAAGCAGGAAAGGGCC | |
| q-*PA4929*-R | CCTCGCTCAACTCGTTGGTA | |
| q-*bifA*-F | GAAGATCACCCTGGACACCG | |
| q-*bifA*-R | GGTAGACCAGGAACAGCACC | |
| q-*PA2072*-F | TTCTACGTGGAAAAGGCGCT | |
| q-*PA2072*-R | CGACCCGGTTTCCCAGATAG | |
| q-*PA3825*-F | TTCGAACGGATGCTCGACAA | |
| q-*PA3825*-R | CGTATTGTCGCGCGTCAAAT | |
| *rplU*-F | GCAGCACAAAGTCACCGAAG | Internal control |
| *rplU*-R | CCGATTTTCACGTCTTCGCC | |
| For EMSA: | | |
| EMSA-*fleSR*-F | AAGGCCTGGACCTCAAGGAC | |
| EMSA-*fleSR*-R | GCTGGTTGCATTGCGTTTC | |
| EMSA-*siaA*-F | AAGACGTGCTGCCGCTCGAA | |
| EMSA-*siaA*-R | GCCATGGCTATCCCTATCAGT | |
| EMSA-*gcbA*-F | CAGCTCGATAGATGGGGGATTG | |
| EMSA-*gcbA*-R | GACGCGCTTCTTTCGTGGTC | |
| EMSA-*sadC*-F | GTGTTGTCCTTGGTGTTCTTCCG | |
| EMSA-*sadC*-R | TCCTACTACCACCCGGTCGAT | |

(Continued on next page)

**TABLE 2** (Continued)

| Primers | Sequence (5′ to 3′) | Description |
|---|---|---|
| EMSA-*mucR*-F | CACCTCCTGTCGAGACATTCAGA | |
| EMSA-*mucR*-R | GAAAGCGTCCGAACGGGATAG | |
| EMSA-*PA4929*-F | CCGAGCTTCATGAAGTCGTCC | |
| EMSA-*PA4929*-R | AGTGAAAACGTCGGAATTGCTC | |
| EMSA-*amrZ*-R | CAATCGGTTGCACGAAGACG | |
| EMSA-*amrZ* -R | AGTTGCCTGTTTCAGTGGGC | |

Proteins were detected using the ECL kit (Abbkine, USA) according to the manufacture's protocol. The RNA polymerase $\beta$ subunit ($\beta$-RNAP) was selected as an internal control for Western blot assays.

**Electrophoretic gel mobility shift assay.** The DNA probes used for EMSA were prepared by PCR using the primer pairs listed in Table 2. The purified PCR products were 3′-end labeled with biotin following the manufacturer's instruction (Thermo Fisher Scientific, USA). The DNA-protein binding reactions were performed according to the manufacturer's instructions (Thermo Fisher Scientific, USA). The 4% polyacrylamide gel was used to separate the DNA-protein complexes. After UV cross-linking, the biotin-labeled probes were detected using a biotin luminescent detection kit (Thermo Fisher Scientific, USA).

**Bacterial killing assay.** The antibacterial activity of *P. aeruginosa* was examined following the method as described by Han et al. (7). *E. coli* cells containing the pBBR1-MCS5 plasmid (gentamicin resistance) served as the prey. Overnight cultures of *P. aeruginosa* and *E. coli* were washed three times with fresh LB and diluted to $OD_{600}$ = 2.0 and 0.4, respectively. Then the two bacteria were 1:1 mixed and 5 $\mu$l of the mixture was spotted on a 0.22-$\mu$m nitrocellulose membrane and placed on an LB agar plate. After incubation at 37°C for 12 h, bacterial cells were re-suspended in 500 $\mu$l LB broth. The cell suspension was serially diluted and spread on LB agar plates containing gentamicin to select for *E. coli* cells. The number of recovered *E. coli* cells was counted.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.8 MB.

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We declare that they have no conflicts of interest with the contents of this article.

T.Z., Z.X., and L.Z. designed the study. T.Z., J.H., Z.L., and Q.L. performed experiments. T.Z., J.H., and Z.X. analyzed the results. T.Z., Z.X., and L.Z. drafted and revised the manuscript.

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