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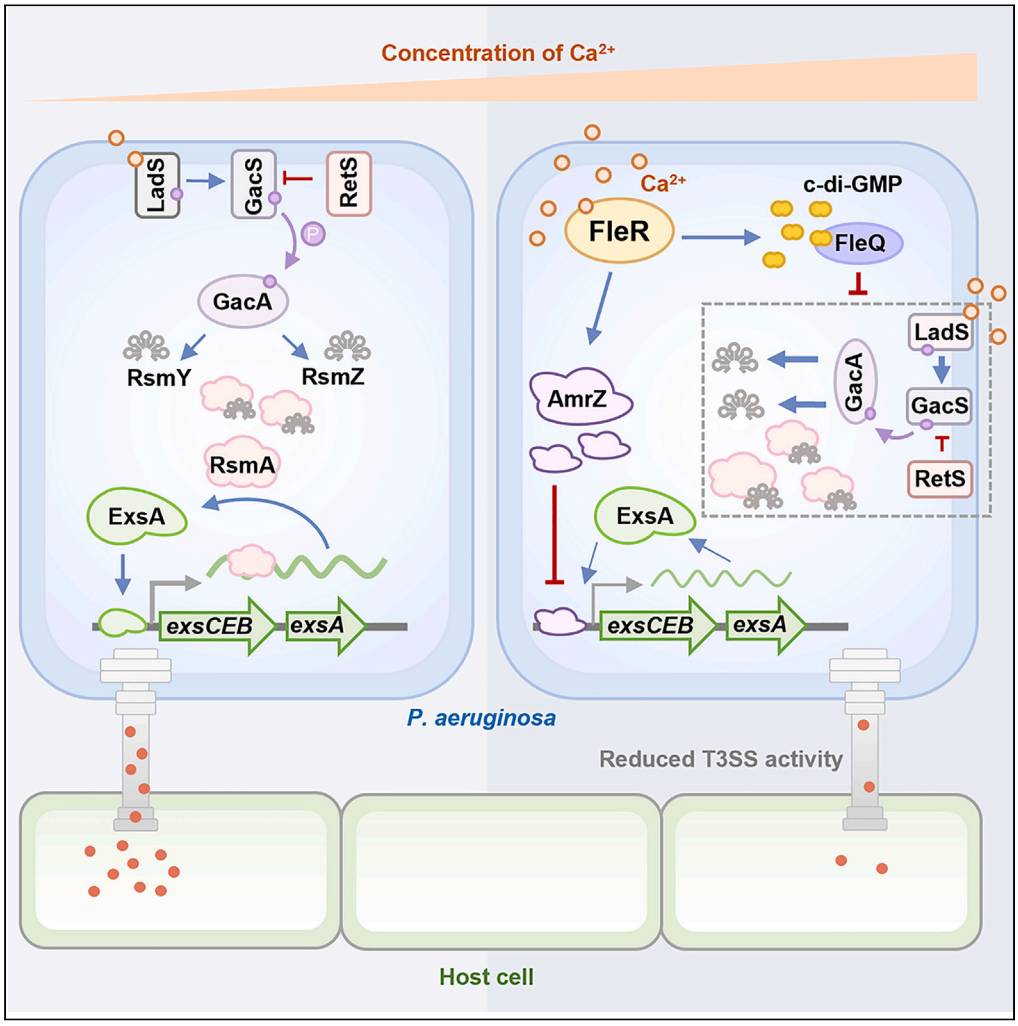

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Highlights

FleR negatively regulates T3SS gene expression and virulence of *P. aeruginosa*

FleR controls T3SS gene expression under Ca^{2+}-rich conditions

FleR regulates T3SS gene expression through the c-di-GMP/FleQ and Gac/Rsm pathways

AmrZ inhibits T3SS gene expression by directly targeting the promoter of *exsCEBA*

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Suppression of *Pseudomonas aeruginosa* type III secretion system by a novel calcium-responsive signaling pathway

Jiahui Huang,[1] Zirui Xu,[1] Tian Zhou,[1] Lian-Hui Zhang,[1,2,*] and Zeling Xu[1,3,*]

SUMMARY

Expression of the type III secretion system (T3SS) in *Pseudomonas aeruginosa* is exquisitely controlled by diverse environmental or host-related signals such as calcium (Ca^{2+}), however, the signal transduction pathways remain largely elusive. In this study, we reported that FleR, the response regulator of the two-component system FleS/FleR, inhibits T3SS gene expression and virulence of *P. aeruginosa* uncoupled from its cognate histidine kinase FleS. Interestingly, FleR was found to repress T3SS gene expression under Ca^{2+}-rich conditions independently of its DNA-binding domain. FleR activates the elevation of intracellular c-di-GMP contents and FleQ serves as the c-di-GMP effector to repress T3SS gene expression through the Gac/Rsm pathway. Remarkably, we found that AmrZ, a member of the FleR regulon, inhibits T3SS gene expression by directly targeting the promoter of *exsCEBA* in an expression level-dependent manner. This study revealed an intricate regulatory network that connects *P. aeruginosa* T3SS gene expression to the Ca^{2+} signal.

INTRODUCTION

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen that is responsible for numerous nosocomial infections.[1] It is one of the leading causes of morbidity and mortality in immunocompromised patients who suffer from cystic fibrosis, burn wounds, cancer, ventilator-associated pneumonia, etc.[2] The pathogenesis of *P. aeruginosa* is related to the abundant virulence factors it produces.[3] Among them, the type III secretion system (T3SS), which consists of five components, i.e., the needle complex, the translocation apparatus, chaperones, effectors, and regulatory proteins, is known to be the key virulence factor employed by *P. aeruginosa* to directly inject effector proteins into host cells, interfering with host physiological processes and establishing severe acute infections.[4,5]

T3SS gene expression in *P. aeruginosa* is associated with a variety of host and environmental signals such as the presence of serum, calcium (Ca^{2+}), spermidine, or host cells. They tightly control T3SS gene expression through a complicated regulatory network involving an intrinsic regulator and numerous extrinsic regulators.[6–11] The intrinsic AraC/XylS-family regulator ExsA is known as the master T3SS activator, which controls the expression of almost all T3SS genes in a partner-switching mechanism with three additional proteins ExsC, ExsD, and ExsE.[12,13] Under non-inducing conditions, intracellular accumulation of the T3SS substrate ExsE binds to its partner, the anti-anti-activator ExsC, which releases the anti-activator ExsD to sequester ExsA and leads to the T3SS genes in a quiescent state.[14,15] When the ExsE is secreted under T3SS-inducing conditions such as contact with host cells or the Ca^{2+}-depleted environment, free ExsC interacts with ExsD and releases ExsA to activate T3SS gene expression.[16,17] The partner-switching regulatory cascade plays a critical role in mediating the bistable expression of T3SS genes since small changes in the levels of ExsA, ExsD, ExsC, and ExsE can have a significant impact on T3SS gene expression.[18,19]

Expression of the intrinsic regulator ExsA is controlled by multiple extrinsic regulators at both the transcriptional and translational levels. Two promoters drive the transcription of *exsA*. One is P*exsA*, which produces a monocistronic *exsA* mRNA, and the other is P*exsCEBA*, which produces a polycistronic *exsCEBA* mRNA. Of these, P*exsCEBA* contributes more to the transcription of *exsA* than the P*exsA* promoter.[20] So far, multiple transcription factors such as the global virulence factor regulator Vfr, the nucleoid-associated protein Fis, the AraC-family transcriptional regulator VqsM, and the histone-like proteins MvaT and MvaU have been identified to regulate *exsA* by binding directly to the promoter P*exsA*.[20–23] However, transcription factors that directly interact with the P*exsCEBA* promoter remain poorly understood. It is known that the activity of P*exsCEBA* is primarily activated by ExsA in a positive feedback loop, and to date, only a few proteins such as a TetR-family protein PsrA and the histone-like protein MvaT have been identified to regulate P*exsCEBA* by directly targeting the P*exsCEBA* promoter.[24,25]

The CsrA-family protein RsmA is essential for stimulating the translation of ExsA.[26] The activity of RsmA is influenced by two small RNAs RsmY and RsmZ which sequester RsmA from target mRNAs, and the expression of RsmY and RsmZ is further regulated by a two-component

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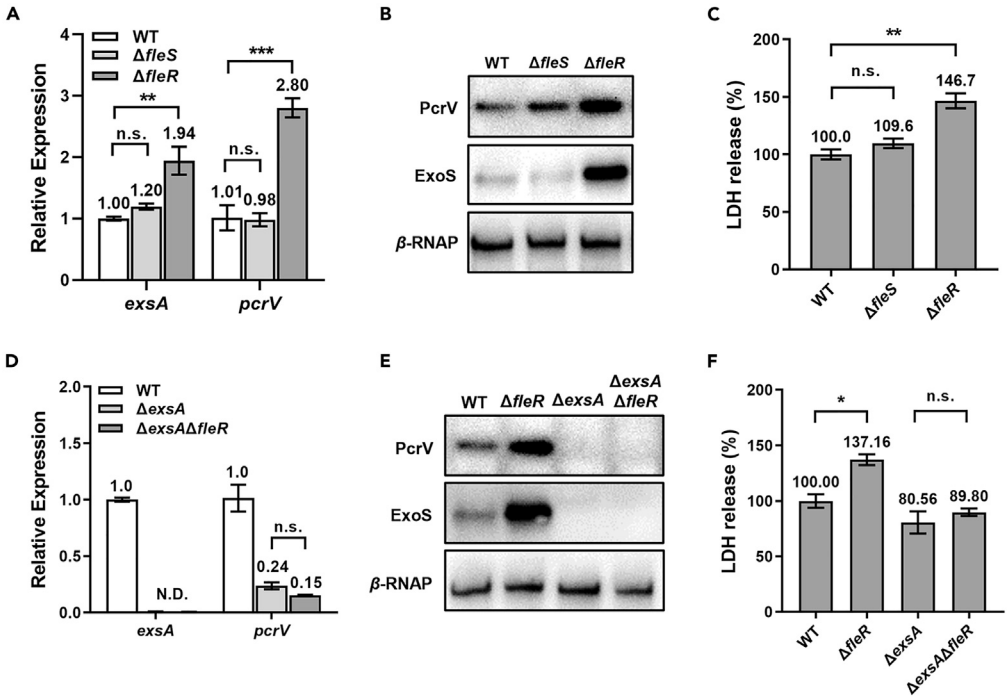

Figure 1. FleR represses T3SS gene expression and bacterial virulence through ExsA

(A) Relative expression of *exsA* and *pcrV* in the Δ*fleS* and Δ*fleR* mutants compared to the PAO1 wild-type (WT) strain was determined by RT-qPCR. The 50S ribosomal protein gene *rplU* was used as an internal control.

(B) Production of T3SS proteins PcrV and ExoS in PAO1 WT, Δ*fleS* and Δ*fleR* strains was measured by western blot assay. The *β*-RNA polymerase (*β*-RNAP) was used as a loading control.

(C) Relative cytotoxicity was assessed by monitoring LDH release from the A549 cells which were infected by the strains of PAO1 WT, Δ*fleS* and Δ*fleR*.

(D) Relative expression of *exsA* and *pcrV* in the Δ*exsA* and Δ*exsA*Δ*fleR* strains was quantified by RT-qPCR. N.D., not detected.

(E) Production of PcrV and ExoS was determined by western blot assay in PAO1 WT, Δ*fleR*, Δ*exsA*, and Δ*exsA*Δ*fleR* strains.

(F) Relative cytotoxicity was assessed by monitoring LDH release from the A549 cells which were infected by the strains of PAO1 WT, Δ*fleR*, Δ*exsA* and Δ*exsA*Δ*fleR*. Statistical analysis was performed using Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n.s., no significance compared to the indicated group.

system (TCS) consisting of the response regulator GacA and the cognate histidine kinase GacS.[27] The Gac/Rsm system is inversely regulated by two additional kinases LadS and RetS.[28] In addition, second messengers such as cyclic AMP (cAMP) and cyclic di-GMP (c-di-GMP) also affect the expression of T3SS genes. Elevated cAMP levels positively regulate T3SS gene expression through the regulator Vfr, whereas elevated c-di-GMP levels negatively regulate T3SS gene expression with unclear mechanisms.[29–31] As the activity of TCSs and intracellular levels of second messengers are often in response to extracellular signals, they are important in linking T3SS gene expression to diverse environmental signals.

The TCS FleS/FleR, consisting of the histidine kinase FleS and the response regulator FleR, regulates multiple virulence-related traits in *P. aeruginosa*.[32–36] Our previous transcriptome analysis showed that the deletion of *fleR* also resulted in the up-regulation of T3SS genes,[33] meaning that FleS/FleR may be a novel regulator of T3SS gene expression. In the present study, we aimed to further investigate the molecular mechanisms underlying the control of T3SS gene expression by the TCS FleS/FleR and reported an interesting and complicated regulatory network in which the response regulator FleR represses T3SS gene expression independently of its DNA-binding domain and the cognate histidine kinase FleS under Ca^{2+}-rich conditions.

RESULTS

FleR is a novel repressor inhibiting T3SS gene expression and virulence in *P. aeruginosa*

To investigate whether T3SS gene expression is controlled by the TCS FleS/FleR, we first collected *P. aeruginosa* PAO1 wild-type (WT), Δ*fleS* and Δ*fleR* strains when they were cultured to OD_{600} of 1.0 for gene expression analysis. Consistent with the previous transcriptomic data,[33] RT-qPCR assay showed that deletion of *fleR* led to a significant increase in mRNA levels of the *exsA* gene and the *pcrV* gene, which encode the master T3SS activator and a component of the translocation apparatus, respectively (Figure 1A). Unexpectedly, the mRNA levels of both T3SS genes were not influenced by the deletion of *fleS* (Figure 1A), suggesting that the response regulator FleR, but not the histidine kinase FleS, negatively regulates T3SS gene expression. To validate this result, we examined the protein levels of PcrV and a secreted toxin protein ExoS in the PAO1 WT, Δ*fleS* and Δ*fleR* strains. Consistent with the RT-qPCR results, western blot assay showed that the amount of both PcrV and ExoS

proteins were obviously increased in the Δ*fleR* mutant (Figure 1B). In contrast, both proteins were produced at the same level in the PAO1 WT and Δ*fleS* strains (Figure 1B). Because T3SS-mediated injection of toxins into human lung epithelial A549 cells is cytotoxic and causes lactate dehydrogenase (LDH) release from the lysed cells,[37] we then evaluated the T3SS activity and bacterial cytotoxicity levels of PAO1 WT, Δ*fleS* and Δ*fleR* strains by infecting the A549 cells with these strains and monitoring LDH release after infection. As shown in Figure 1C, deletion of *fleR* but not *fleS* resulted in significantly increased cytotoxicity compared to the WT strain. Taken together, these results demonstrated that the response regulator FleR from the TCS FleS/FleR represses T3SS gene expression and virulence in *P. aeruginosa*.

Owing that ExsA is the master activator of the entire T3SS in *P. aeruginosa* and its expression was up-regulated in the Δ*fleR* mutant (Figure 1A), we wondered whether FleR represses T3SS gene expression by repressing ExsA. We therefore examined T3SS gene expression in the Δ*exsA* mutant. Compared to the PAO1 WT strain, deletion of *exsA* resulted in undetectable *exsA* mRNA and a substantial decrease in the mRNA level of *pcrV* (Figure 1D). However, in contrast to the WT strain, further deletion of *fleR* in Δ*exsA* did not elevate the mRNA level of *pcrV* (Figure 1D), suggesting that *exsA* is required for the higher T3SS gene expression in the Δ*fleR* mutant. Consistently, western blot assay showed that the production of T3SS-associated proteins was no longer induced by the deletion of *fleR* in the Δ*exsA* mutant (Figure 1E), confirming the central role of ExsA in mediating the up-regulation of T3SS gene expression in the Δ*fleR* mutant. Moreover, LDH release assay confirmed that bacterial cytotoxicity was also no longer induced by the deletion of *fleR* in the Δ*exsA* mutant (Figure 1F). Therefore, FleR acts as a novel extrinsic repressor of T3SS and inhibits *P. aeruginosa* virulence via ExsA.

FleR uncouples from FleS to negatively regulate T3SS gene expression by repressing the transcription of *exsCEBA*

The transcription of *exsA* is driven by two promoters, i.e., P*exsCEBA* and P*exsA*. We then investigated which promoter was activated to induce *exsA* in Δ*fleR*. P*exsCEBA-lacZ* and P*exsA-lacZ* transcriptional fusions were used for this investigation and β-galactosidase activity assay showed that the promoter activity of *exsCEBA* was significantly higher in the Δ*fleR* mutant than that in the WT strain, whereas the promoter activity of *exsA* was similar in both strains (Figure 2A). Chromosomal complementation of *fleR* in the Δ*fleR* mutant significantly reduced the P*exsCEBA* activity almost back to the WT level (Figure S1). Further deletion of *fleR* in Δ*exsA* did not increase the promoter activity of *exsCEBA* and *in trans* overexpression of *exsA* in the mutant of Δ*exsA*Δ*fleR* restored the promoter activity of *exsCEBA* to a high level (Figure 2B). These results together demonstrated that FleR represses the expression of *exsA* by inhibiting the *exsCEBA* promoter indirectly through ExsA.

FleS and FleR were reported to regulate biofilm formation together in a coupled TCS signaling pattern. Like other canonical TCS partners, the aspartate residue at position 53 (D53) in the REC domain of FleR was known to be a conserved and essential residue for receiving the phosphoryl group and signaling.[33,36] If FleR regulates T3SS gene expression independently of FleS, we reasoned that disrupting the signaling between FleS and FleR by mutating the aspartate residue D53 in FleR would not affect its function in repressing T3SS gene expression. To test this, we introduced the wild-type FleR and its D53A variant, in which the 53rd aspartate residue was replaced by an alanine (A), into the Δ*fleR* mutant. It was shown that *in trans* overexpression of either WT FleR or FleR^{D53A} under the control of a constitutive promoter in the Δ*fleR* mutant substantially reduced the promoter activity of *exsCEBA* to a level that was approximately 70% lower than that in the WT strain (Figure 2C). This result not only indicated that FleR^{D53A} remains functional to repress T3SS but also showed that the repression intensity of T3SS is associated with the expression level of FleR. We also examined other FleR variants carrying an aspartate to alanine substitution at all the aspartate sites within the REC domain besides D53. β-galactosidase activity assay and western blot assay showed that all the FleR variants were still functional as the WT FleR to inhibit T3SS gene expression in the Δ*fleR* mutant (Figure S2). In addition, *in trans* overexpression of FleR in the Δ*fleS* mutant significantly inhibited the promoter activity of *exsCEBA* (Figure 2D), further confirming that FleR regulates T3SS gene expression independently of its cognate histidine kinase FleS.

FleR represses T3SS gene expression in response to extracellular Ca^{2+}

FleR consists of three domains: REC, AAA and HTH (Figure 3A). Because FleR regulates T3SS gene expression independently of FleS, we were motivated to re-evaluate the contribution of each domain of FleR to the regulation of T3SS gene expression. We introduced FleR and its ΔREC, ΔAAA, and ΔHTH variants into the Δ*fleR* mutant and found that *in trans* overexpression of the FleRΔREC and FleRΔAAA variants only slightly reduced the promoter activity of *exsCEBA*, whereas the FleR WT and the FleRΔHTH variant could substantially inhibit the promoter activity of *exsCEBA* (Figure 3B). This result suggests that the REC and AAA domains are essential while the HTH domain is dispensable for FleR to regulate T3SS gene expression.

T3SS gene expression is controlled by numerous environmental cues and the response regulator plays a critical role in signaling. We next moved to explore which environmental cues are transduced by FleR to repress T3SS gene expression. The crystal structure of the FleR REC domain was recently solved (PDB: 7W9H),[38] and interestingly, a Ca^{2+} ligand was found to interact with the REC domain instead of a Mg^{2+} ion, which is highly conserved in the REC domain of response regulators for the catalysis of phosphorelay reactions.[39] Ca^{2+} is an important host-related signal that triggers the acute-to-chronic virulence switch in *P. aeruginosa*, and repression of T3SS genes correlates with high Ca^{2+} concentrations.[40] Since the up-regulation of T3SS genes in the Δ*fleR* mutant was observed under normal growth condition without removal of Ca^{2+} from the medium in this study, we were curious whether FleR mediates T3SS gene repression in response to Ca^{2+}. To test this speculation, we used Ca^{2+}-chelating reagents to deplete free Ca^{2+} ions in the medium. As shown in Figures 3C and 3D, supplementation of the Ca^{2+}-chelating reagents ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) or nitrilotiracetic acid (NTA) in the culture medium induced the P*exsCEBA* activity. Meanwhile, we found that the increased intensity of the P*exsCEBA* activity due to *fleR* deletion was diminished with increasing concentrations of both Ca^{2+}-chelating reagents (Figures 3C and 3D). These results displayed that FleR represses T3SS gene expression under Ca^{2+}-rich conditions.

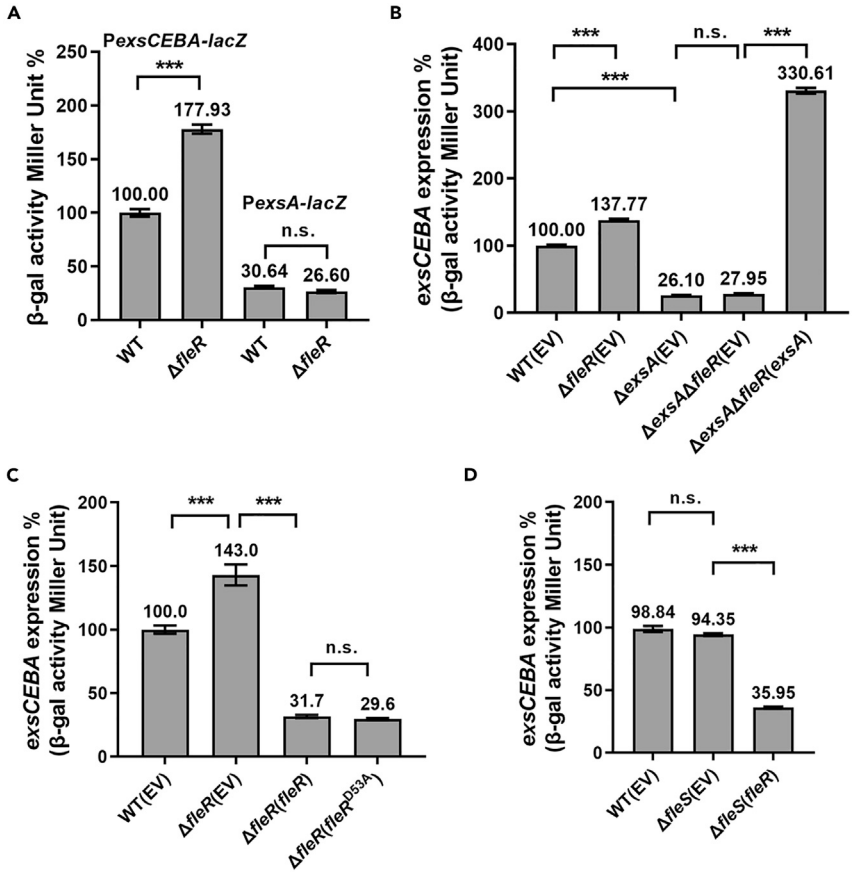

Figure 2. FleR inactivates the transcriptional activity of the *exsCEBA* promoter independently of FleS

(A) β-galactosidase activity of the P*exsCEBA-lacZ* and P*exsA-lacZ* transcriptional fusions was measured in the PAO1 WT and Δ*fleR* strains.

(B) β-galactosidase activity of the P*exsCEBA-lacZ* transcriptional fusion was measured in PAO1 WT, Δ*fleR*, Δ*exsA*, Δ*exsA*Δ*fleR*, and Δ*exsA*Δ*fleR*(*exsA*) strains. EV represents empty vector.

(C) β-galactosidase activity of the P*exsCEBA-lacZ* transcriptional fusion was measured in the Δ*fleR* mutant with *in trans* overexpression of FleR and its D53A variant.

(D) β-galactosidase activity of the P*exsCEBA-lacZ* transcriptional fusion was measured in the Δ*fleS* mutant with *in trans* overexpression of FleR. Statistical analysis was performed using Student's *t* test. ***, $p < 0.001$; n.s., no significance compared to the indicated group.

FleR induces c-di-GMP production to repress T3SS gene expression

Our previous studies have shown that FleR induces *P. aeruginosa* biofilm formation and represses type VI secretion system (T6SS) by elevating intracellular c-di-GMP levels.[33,36] Therefore, we wanted to explore whether c-di-GMP also mediates T3SS gene repression for FleR. Since c-di-GMP levels in the PAO1 WT and Δ*fleR* strains were indirectly assessed using a biosensor in previous studies, here we further confirmed that FleR positively regulates the intracellular level of c-di-GMP by measurement using LC-MS in the PAO1 WT, Δ*fleR* strains, and the Δ*fleR* strain with chromosomal complementation of *fleR* (Figure 4A).

To examine whether increased c-di-GMP mediates the repression of T3SS for FleR, we introduced two genes, *W909_14945* (14945) and *W909_14950* (14950), into the Δ*fleR* mutant. 14945 encodes a diguanylate cyclase for c-di-GMP biosynthesis, and 14950 encodes a phosphodiesterase for c-di-GMP hydrolysis from *Dickeya zeae* EC1.[41] Previous studies have demonstrated that expression of two genes is effective in increasing and decreasing intracellular c-di-GMP levels in *P. aeruginosa*, respectively.[36,42] Next, β-galactosidase activity assay using the P*exsCEBA-lacZ* transcriptional fusion showed that the induced transcriptional activity of the *exsCEBA* promoter in Δ*fleR* was significantly decreased by expressing the diguanylate cyclase 14945 in the cell (Figure 4B). Consistently, both the mRNA and protein levels of ExsA in Δ*fleR* were reduced to almost WT levels when the diguanylate cyclase 14945 was expressed in the cell (Figures 4C and 4D). As a control, expression of the phosphodiesterase 14950 in Δ*fleR* did not reduce the transcription and translation of the *exsA* gene (Figures 4B–4D). Together, these results indicated that FleR represses T3SS gene expression through the c-di-GMP signaling pathway.

c-di-GMP represses T3SS gene expression through its effector protein FleQ

Bacterial physiological changes in response to the intracellular levels of the second messenger c-di-GMP are mediated by a group of effector proteins that interact with the c-di-GMP molecule.[43] It was previously reported that the transcription factor FleQ is a c-di-GMP responsive

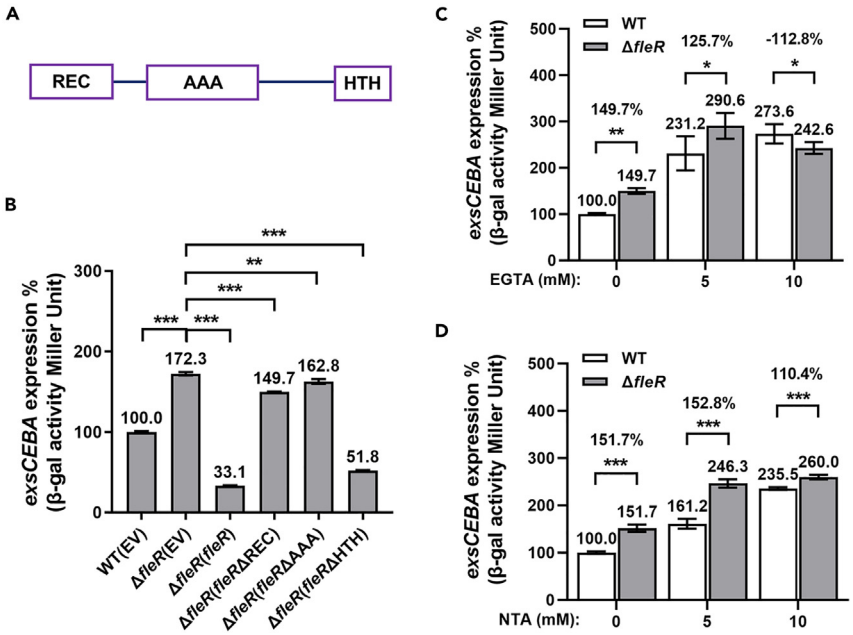

Figure 3. FleR modulates the transcription of P*exsCEBA* under Ca^{2+}-rich conditions

(A) A diagram showing the domain structure of the FleR protein.

(B) β-galactosidase activity of the P*exsCEBA-lacZ* transcriptional fusion was measured in the strains of PAO1 WT, Δ*fleR* and Δ*fleR* with *in trans* overexpression of FleR or its variants with the absence of REC, AAA or HTH domain.

(C) β-galactosidase activity of the P*exsCEBA-lacZ* transcriptional fusion was measured in PAO1 WT and Δ*fleR* strains with the supplementation of EGTA in the growth medium.

(D) β-galactosidase activity of the P*exsCEBA-lacZ* transcriptional fusion was measured in PAO1 WT and Δ*fleR* strains with the supplementation of NTA in the growth medium. Statistical analysis was performed using one-way ANOVA or Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to the indicated group.

effector that positively regulates *P. aeruginosa* motility and biofilm formation, respectively, at low and high levels of c-di-GMP.[44] As we have previously shown the importance of FleQ in mediating c-di-GMP to regulate biofilm formation and T6SS gene expression in *P. aeruginosa*,[33,36] we next sought to know whether FleQ also mediates the regulation of T3SS gene expression. β-galactosidase activity assay showed that deletion of *fleQ* in the WT strain induced the transcriptional activity of the *exsCEBA* promoter and additional deletion of *fleR* no longer increased the transcriptional activity of *exsCEBA* in the Δ*fleQ* mutant (Figure 5A). Western blot assay confirmed that deletion of *fleQ* increased the production of ExsA in the WT strain and further deletion of *fleR* in the Δ*fleQ* background did not cause any difference in ExsA production (Figures 5B and 5C). These results suggested that FleQ is a potentially key factor connecting T3SS gene expression to FleR and c-di-GMP.

To test whether c-di-GMP regulates T3SS gene expression through the effector protein FleQ, we introduced 14945 and 14950 into the Δ*fleQ* mutant to increase and decrease intracellular c-di-GMP levels, respectively, and then measured the translational activity of ExsA. Western blot assay confirmed that deletion of *fleQ* led to a substantial elevation of the ExsA protein (Figures 5D and 5E). In contrast to the Δ*fleR* mutant, which reduced ExsA production by increasing the intracellular c-di-GMP levels (Figure 4D), increasing intracellular c-di-GMP levels by expressing 14945 in the Δ*fleQ* mutant was unable to alter the protein level of ExsA (Figures 5D and 5E). Moreover, the production of ExsA was not significantly altered when 14945 was expressed in the Δ*fleQ* Δ*fleR* mutant (Figures 5F and 5G).

Considering that residues such as R144, R185, N186, E330, and R334 in FleQ have been reported to coordinate the second messenger c-di-GMP,[45] we next replaced these residues with alanine (A) to see if disrupting the interaction between FleQ and c-di-GMP would affect the regulation of T3SS gene expression. As shown in Figure 5H, *in trans* overexpression of FleQ significantly reduced the P*exsCEBA* activity in the Δ*fleQ* mutant. However, substitutions of residues involved in c-di-GMP interaction alleviated the ability of FleQ to inhibit P*exsCEBA* activity. When R144, N186, and E330 were replaced by alanine, the FleQ activity of T3SS gene repression was almost completely abolished (Figure 5H). All together, these results demonstrated that FleR induces the intracellular level of c-di-GMP to repress T3SS gene expression through FleQ.

FleR and FleQ regulate T3SS gene expression via the Gac/Rsm pathway

Although it remains largely unclear how c-di-GMP controls T3SS gene expression, interactions between c-di-GMP and other important T3SS regulatory systems such as the Gac/Rsm and cAMP/Vfr systems have been reported.[30,46] Since Vfr directly targets the P*exsA* promoter and the activity of this promoter was not changed by the deletion of *fleR* (Figure 2A), we next investigated whether FleR regulates T3SS gene expression by interacting with the Gac/Rsm pathway. The Gac/Rsm pathway is inversely regulated by the hybrid sensor histidine kinases LadS and

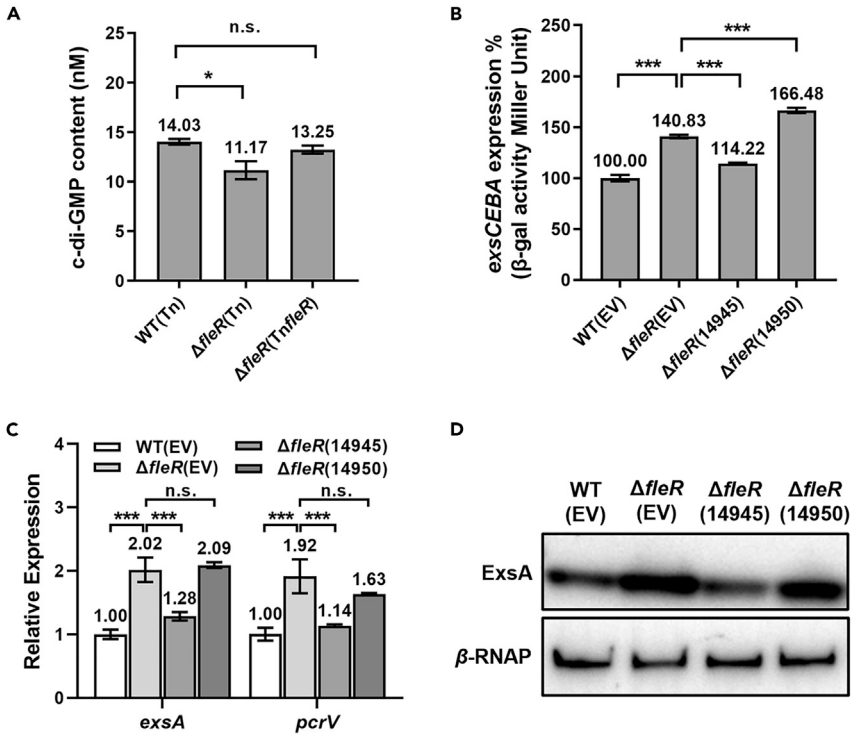

Figure 4. FleR downregulates T3SS gene expression by elevating the c-di-GMP level

(A) Intracellular c-di-GMP contents in the strains of PAO1 WT, Δ*fleR* and Δ*fleR* with chromosomal complementation of *fleR* were measured by LC-MS. Tn: empty control of the Tn7 vector.

(B) *β*-galactosidase activity of the P*exsCEBA-lacZ* transcriptional fusion was measured in PAO1 WT(EV), Δ*fleR*(EV), Δ*fleR*(14945), and Δ*fleR*(14950) strains.

(C) Relative expression of *exsA* and *pcrV* in PAO1 WT(EV), Δ*fleR*(EV), Δ*fleR*(14945), and Δ*fleR*(14950) strains was measured by RT-qPCR.

(D) Production of the master T3SS activator ExsA in PAO1 WT(EV), Δ*fleR*(EV), Δ*fleR*(14945), and Δ*fleR*(14950) strains was measured by western blot assay. Statistical analysis was performed using Student's *t* test. *, $p < 0.05$; ***, $p < 0.001$; n.s., no significance compared to the indicated group.

RetS, which are located at the top of the Gac/Rsm pathway (Figure 6A). To assess whether the Gac/Rsm pathway is involved in T3SS repression, the expression of *ladS* and *retS* was measured using the promoter-*gfp* fusions. It showed undetectable differences in the GFP fluorescence between the WT strain and the Δ*fleR* mutant (Figures S3A and S3B). Interestingly, when we deleted *ladS* and *retS* to activate and inactivate the T3SS gene expression, respectively, it was shown that further deletion of *fleR* in the Δ*ladS* and Δ*retS* background increased the promoter activity of *exsCEBA* with much lower levels (112.54% and 113.55%) than that in the WT background (Figures S4A and S4B). Moreover, *in trans* overexpression of *ladS* and *retS* in the Δ*ladS* Δ*fleR* and Δ*retS* Δ*fleR* mutants decreased and increased the promoter activity of *exsCEBA*, respectively (Figures S4A and S4B). These results implied that the Gac/Rsm pathway may be necessary for the up-regulation of T3SS gene expression upon the deletion of *fleR*, as the up-regulation of T3SS gene expression was greatly attenuated when it was already highly active or inactive due to the loss of LadS or RetS.

We next monitored the expression of *rsmA*, *rsmY*, and *rsmZ* which directly control target gene expression in the Gac/Rsm pathway, but there was still no difference in their expression between the WT strain and the Δ*fleR* mutant (Figure S3C). Since RsmA is the terminal effector in the Gac/Rsm pathway that activates ExsA translation (Figure 6A), we deleted *fleR* in the Δ*rsmA* background and sought to examine the role of RsmA in T3SS gene regulation in the Δ*fleR* mutant. As shown in Figure 6B, further deletion of *fleR* in the Δ*rsmA* mutant did not elevate the mRNA levels of *exsA* and *pcrV*. Consistently, further deletion of *fleR* did not elevate the activity of the *exsCEBA* promoter in the Δ*rsmA* mutant and *in trans* overexpression of *rsmA* in the Δ*rsmA* Δ*fleR* mutant could restore the activity of the *exsCEBA* promoter (Figure 6C). We also deleted *fleQ* in the Δ*rsmA* background and then measured the activity of the *exsCEBA* promoter. Same as the result of the *fleR* deletion, further deletion of *fleQ* in the Δ*rsmA* mutant was incapable of inducing the activity of the *exsCEBA* promoter and *in trans* overexpression of *rsmA* in the Δ*rsmA* Δ*fleQ* mutant restored the activity of the *exsCEBA* promoter (Figure 6D). All these data indicated that FleR and FleQ regulate T3SS gene expression through the Gac/Rsm pathway.

AmrZ, a member belonging to the FleR regulon, represses T3SS gene expression by directly targeting the *exsCEBA* promoter

Expression of AmrZ, a ribbon-helix-helix transcription factor implicated in repressing T3SS with an undetermined mechanism in *P. aeruginosa*,[47] was significantly reduced in the Δ*fleR* mutant (Figure S5).[33] We next complemented *amrZ* in the Δ*fleR* mutant to investigate

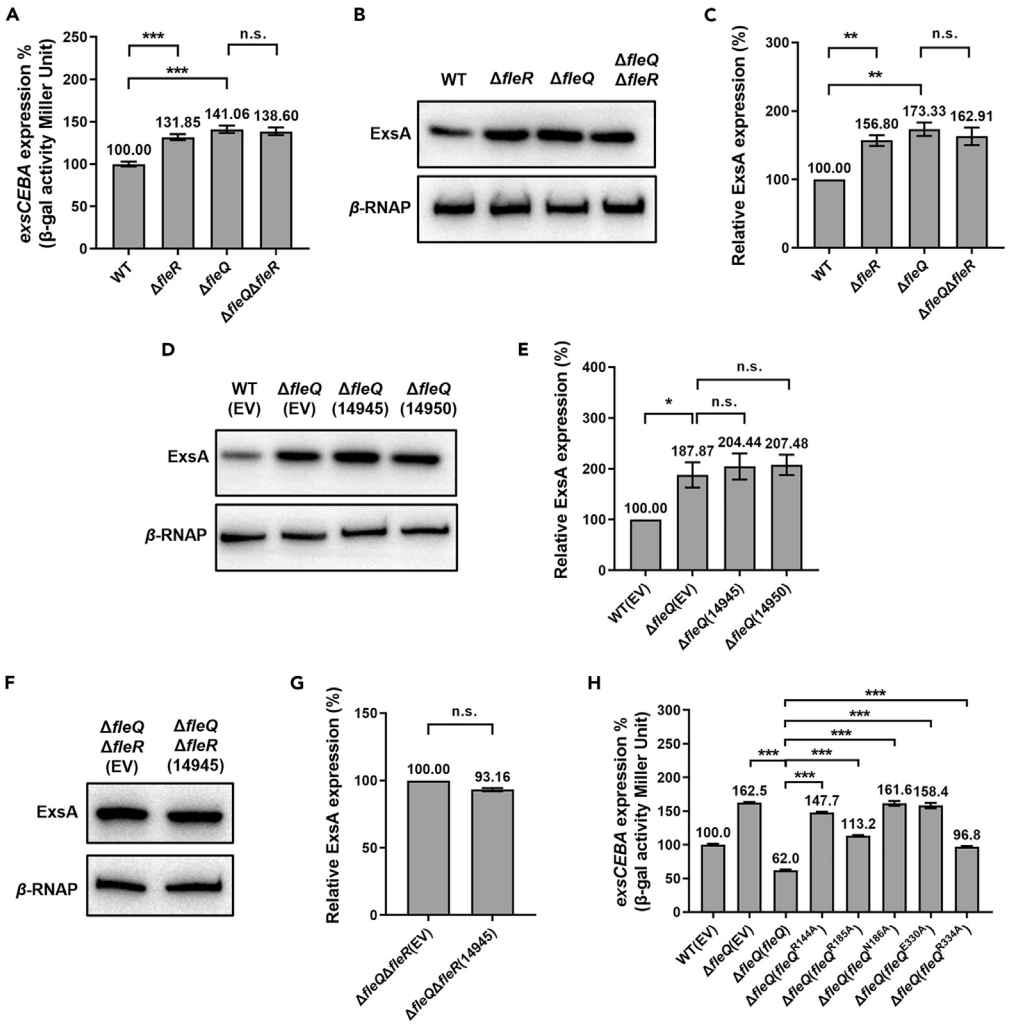

Figure 5. FleR regulates T3SS gene expression through the c-di-GMP effector FleQ

(A) β-galactosidase activity of the P*exsCEBA-lacZ* transcriptional fusion was measured in PAO1 WT, Δ*fleR*, Δ*fleQ*, and Δ*fleQ*Δ*fleR* strains.

(B) Production of ExsA in PAO1 WT, Δ*fleR*, Δ*fleQ*, and Δ*fleQ* Δ*fleR* strains was determined by western blot assay.

(C) Quantification of the ExsA production in (B) and the result was shown as relative ExsA expression to the WT strain.

(D) Production of ExsA in PAO1 WT(EV), Δ*fleR*(EV), Δ*fleQ*(14945), and Δ*fleQ*(14945) strains was determined by western blot assay.

(E) Quantification of the ExsA production in (D) and the result was shown as relative ExsA expression to the WT(EV) strain.

(F) Production of ExsA in Δ*fleQ*Δ*fleR*(EV) and Δ*fleQ*Δ*fleR*(14945) strains was determined by western blot assay.

(G) Quantification of the ExsA production in (F) and the result was shown as relative ExsA expression to the Δ*fleQ*Δ*fleR*(EV) strain.

(H) β-galactosidase activity of the P*exsCEBA-lacZ* transcriptional fusion was measured in the strains of PAO1 WT, Δ*fleQ* and Δ*fleQ* with *in trans* overexpression of FleQ or its variants with residue substitutions at R144, R185, N186, E330, or R334. Statistical analysis was performed using Student's *t* test or one-way ANOVA. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n.s., no significance compared to the indicated group.

if it links T3SS gene expression to FleR. When *amrZ* was constitutively expressed in the Δ*fleR* mutant, it fully abolished the up-regulation of ExsA at both the transcriptional and translational levels (Figures 7A and 7B). Unexpectedly, when we sought to confirm the function of *amrZ* by deleting the gene, we did not observe any change in ExsA production as well as the promoter activity of *exsCEBA* (Figures S6A and S6B), suggesting that the basal expression level of AmrZ did not alter T3SS gene expression and therefore downregulation of *amrZ* in the Δ*fleR* mutant might not influence T3SS gene expression. However, it was shown that the production of ExsA and the promoter activity of *exsCEBA* were substantially inhibited in the WT strain with the constitutive expression of AmrZ (Figures 7B and 7C), implying that AmrZ can repress T3SS genes when it is overproduced, although the basal expression level of *amrZ* has no detectable effect on T3SS gene expression. This result also validated that the up-regulation of T3SS genes due to the deletion of *fleR* is mainly dependent on the c-di-GMP/FleQ and Gac/Rsm pathways.

Considering the regulatory role of AmrZ in modulating c-di-GMP metabolism,[47] we next explored whether or not constitutively expressed AmrZ regulates T3SS gene expression through the c-di-GMP/FleQ pathway or not by expressing AmrZ in the c-di-GMP overproducing and

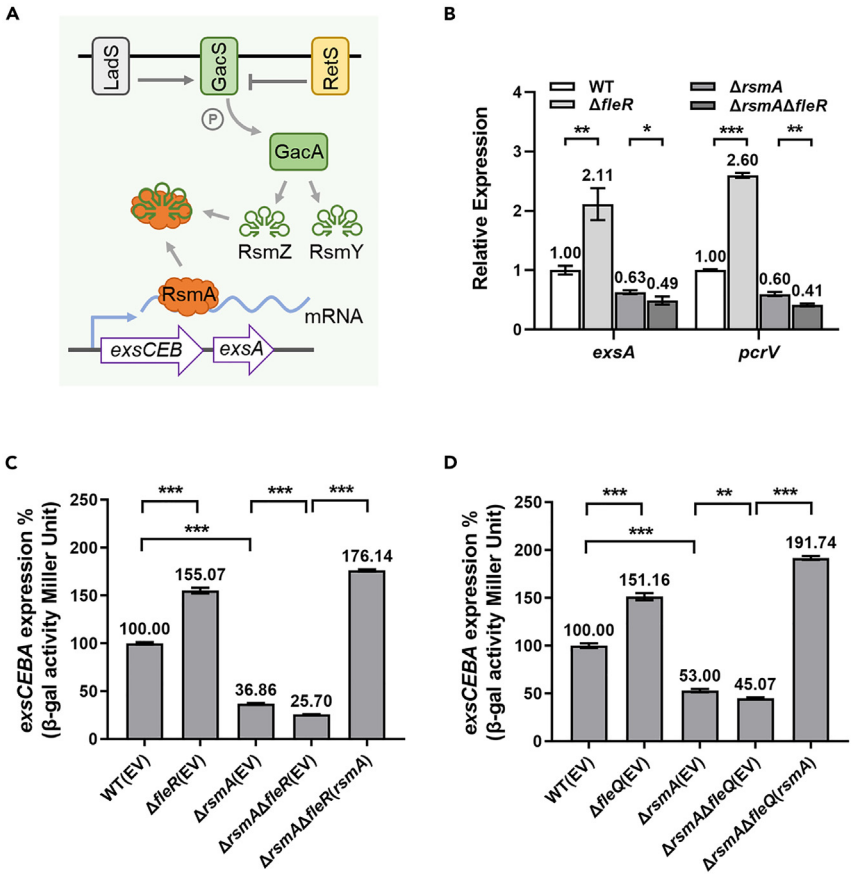

Figure 6. FleR and FleQ regulate T3SS gene expression *via* the Gac/Rsm pathway

(A) A diagram showing the regulatory pattern of T3SS by the Gac/Rsm pathway. RsmA stimulates the translation of ExsA and the activity of RsmA is influenced by two small RNAs RsmY and RsmZ which sequester RsmA from target mRNAs. Expression of RsmY and RsmZ is regulated by a two-component system GacS/GacA which is inversely regulated by two additional kinases LadS and RetS.

(B) Relative expression of *exsA* and *pcrV* in the Δ*rsmA* and Δ*rsmA*Δ*fleR* strains was measured by RT-qPCR.

(C) *β*-galactosidase activity of the P*exsCEBA-lacZ* transcriptional fusion was measured in PAO1 WT, Δ*fleR*, Δ*rsmA*, Δ*rsmA*Δ*fleR*, and Δ*rsmA*Δ*fleR*(*rsmA*) strains.

(D) *β*-galactosidase activity of the P*exsCEBA-lacZ* transcriptional fusion was measured in PAO1 WT, Δ*fleQ*, Δ*rsmA*, Δ*rsmA*Δ*fleQ*, and Δ*rsmA*Δ*fleQ*(*rsmA*) strains. Statistical analysis was performed using Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to the indicated group.

depleted strains. As shown in Figure 7D, constitutive expression of AmrZ significantly inhibited the activity of the *exsCEBA* promoter regardless of the intracellular c-di-GMP levels. Constitutive expression of AmrZ also inhibited the activity of the *exsCEBA* promoter to a similar extent in both the WT strain and the Δ*fleQ* mutant (Figure 7E). These results demonstrated that overproduced AmrZ inhibits T3SS gene expression independently of the c-di-GMP/FleQ pathway.

Since the independence of the c-di-GMP/FleQ pathway and the strong intensity of T3SS gene repression, we speculated that AmrZ might directly target *exsCEBA* or *exsA* promoters to influence *exsA* expression. In addition to the activity of the *exsCEBA* promoter, we also compared the activity of the *exsA* promoter in the PAO1 WT strain, the Δ*amrZ* mutant, and the WT strain with the constitutive expression of *amrZ*. In contrast to the dramatically reduced activity of the *exsCEBA* promoter (Figure 7C), β-galactosidase activity assay showed no difference in the activity of the *exsA* promoter with deletion or constitutive expression of *amrZ* (Figures S6B and 7C), suggesting that AmrZ may target the *exsCEBA* promoter but not the *exsA* promoter. The AmrZ protein was then purified and potential interactions between AmrZ and the two promoters were examined using the electrophoretic mobility shift assay (EMSA). As shown in Figure 7F, AmrZ was able to bind to the *exsCEBA* promoter but not to the *exsA* promoter, demonstrating that AmrZ is a novel T3SS repressor that directly targets the *exsCEBA* promoter to inhibit its transcriptional activity, which relies on a high level of AmrZ being produced in the cell.

DISCUSSION

The TCS FleS/FleR has been demonstrated to play an important role in regulating motility, biofilm formation and T6SS gene expression in *P. aeruginosa*.[33–36] As suggested by the transcriptome result, in this study, we further investigated its role in regulating the expression of T3SS,

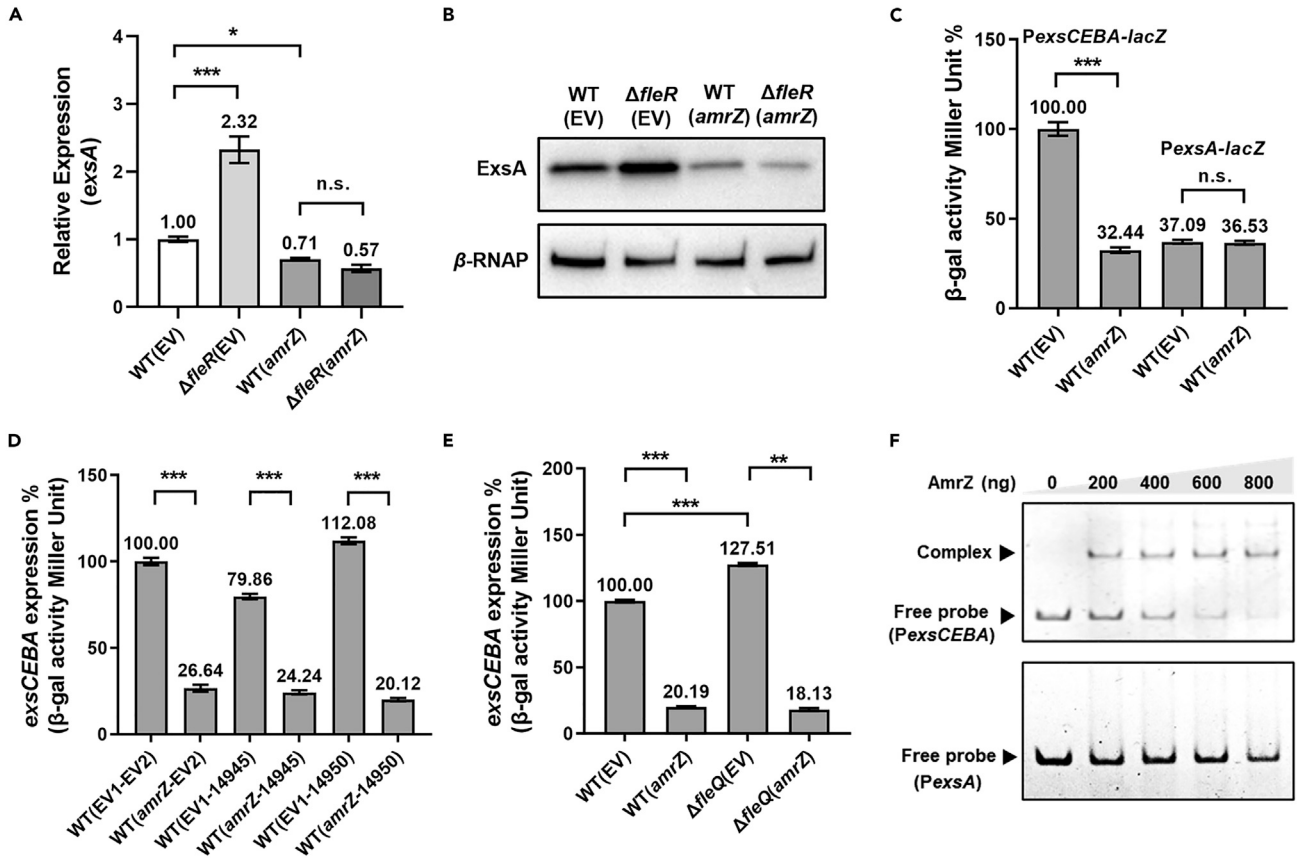

Figure 7. AmrZ directly inhibits the transcription of *exsCEBA* when it is overproduced

(A) Relative expression of *exsA* in the PAO1 WT and Δ*fleR* strains with or without the constitutive expression of *amrZ*.

(B) Production of ExsA in PAO1 WT(EV), Δ*fleR*(EV), Δ*fleR*(*amrZ*), and Δ*fleR*(*amrZ*) strains was determined by western blot assay.

(C) *β*-galactosidase activity of the P*exsCEBA-lacZ* and P*exsA-lacZ* transcriptional fusions was measured in the PAO1 WT strain with or without the constitutive expression of *amrZ*.

(D) *β*-galactosidase activity of the P*exsCEBA-lacZ* transcriptional fusion was measured in the PAO1 WT strain with the co-expression of *amrZ* and 14945 or 14950.

(E) *β*-galactosidase activity of the P*exsCEBA-lacZ* transcriptional fusion was measured in the PAO1 WT(EV), WT(*amrZ*), Δ*fleQ*(EV) and Δ*fleQ*(*amrZ*) strains.

(F) EMSA examination showing the binding of AmrZ to the promoter of *exsCEBA* but not the promoter of *exsA*. Statistical analysis was performed using Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n.s., no significance compared to the indicated group.

a key virulence factor causing acute infections, and discovered that FleR is a novel extrinsic repressor for T3SS gene expression. Interestingly, the response regulator FleR was shown to negatively regulate T3SS gene expression independently of its DNA-binding domain as well as the cognate histidine kinase FleS, and the repressive behavior was activated by the signal Ca^{2+}. Further investigations revealed that FleR represses T3SS gene expression by inhibiting the expression of the master T3SS activator ExsA through the c-di-GMP/FleQ and Gac/Rsm pathways. Importantly, a transcription factor belonging to the FleR regulon, AmrZ, was identified as a novel member that represses T3SS gene expression by directly targeting the promoter of *exsCEBA*, representing a new regulatory pathway controlling T3SS gene expression in parallel with the c-di-GMP/FleQ and Gac/Rsm pathways. In conclusion, this study depicted a novel signaling network that modulates T3SS gene expression in response to the host-related signal Ca^{2+} (Figure 8).

P. aeruginosa T3SS gene expression is primarily controlled by the master activator ExsA and a variety of extrinsic regulators. FleR was identified as a novel extrinsic regulator that controls T3SS gene expression through ExsA by recruiting the c-di-GMP/FleQ and Gac/Rsm pathways. Both FleS and FleR have been shown to promote biofilm formation by elevating intracellular c-di-GMP levels in *P. aeruginosa*.[36] Therefore, it is elusive why only FleR but not FleS was shown to regulate T3SS gene expression, since both the biofilm formation and T3SS suppression were caused by increased c-di-GMP levels. Among the known extrinsic regulatory pathways, the well-characterized Gac/Rsm pathway has previously been reported to respond to the shift of intracellular c-di-GMP levels.[30,48] We confirmed that FleR and the c-di-GMP effector FleQ repress T3SS gene expression through the Gac/Rsm pathway, but the expression of components of this signaling cascade such as *rsmA*, *rsmY*, *rsmZ*, *ladS*, and *retS* were not altered by the deletion of *fleR*. We speculated that signal transduction through the Gac/Rsm pathway might be achieved through protein-protein interactions of FleQ and the terminal

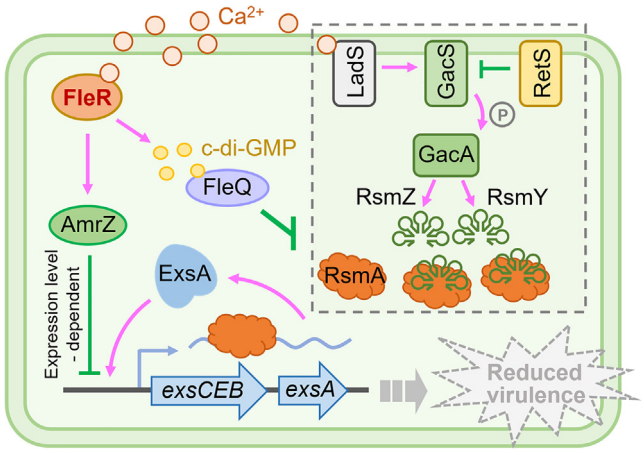

Figure 8. A diagram showing the proposed regulatory pathways that connect *P. aeruginosa* T3SS gene expression to the extracellular signal Ca^{2+}

FleR negatively regulates T3SS gene expression by repressing the master T3SS activator ExsA under Ca^{2+}-rich conditions. When FleR is normally expressed, it reduces T3SS gene expression and virulence of *P. aeruginosa* mainly through the c-di-GMP/FleQ and Gac/Rsm pathways, which is independent of its DNA-binding motif. AmrZ, a transcription factor belonging to the FleR regulon, inhibits T3SS gene expression by directly targeting the promoter of *exsCEBA* in an expression level-dependent manner. The pink arrows indicate activation or positive regulation, whereas green T-shaped symbols indicate inactivation or negative regulation.

effector RsmA in the pathway. However, the pull-down assay did not show any physical interaction between them (Figure S7). Since a recent study reported that FleQ can regulate downstream genes at the post-transcriptional level,[49] it is also possible that FleQ regulates *rsmA* post-transcriptionally.

FleQ is a transcription factor that regulates *P. aeruginosa* motility and biofilm formation. It functions as both a repressor and an activator of biofilm formation in response to varying levels of intracellular c-di-GMP.[50] Binding of c-di-GMP converts FleQ from a repressor to an activator of Pel exopolysaccharide genes and promotes biofilm formation.[50] While for motility, it is recognized as an activator of flagellar gene expression. c-di-GMP binding to FleQ decreases its activity and reduces motility.[51] Although the downstream targets for FleQ that modulate T3SS gene expression remain unclear, our data suggested that FleQ may function solely as a repressor of T3SS genes when it interacts with c-di-GMP, Since T3SS genes were derepressed to a similar extent when the *fleQ* gene was deleted and c-di-GMP levels were reduced by deleting *fleR*. If FleQ was converted into an activator together with the depletion of c-di-GMP, then the Δ*fleR* mutant was expected to exhibit a higher level of T3SS gene expression compared to the Δ*fleQ* mutant. This study added to our knowledge of the function of FleQ in regulating T3SS gene expression.

The master T3SS activator ExsA is transcribed from two promoters, P*exsA* and P*exsCEBA*. In recent years, a number of transcription factors that directly control the activity of P*exsA* have been identified, but little is known about the transcription factors that directly control the activity of P*exsCEBA*.[6] Although AmrZ regulates c-di-GMP metabolism,[47] it directly and robustly represses T3SS gene expression by binding to the promoter of *exsCBEA*. Consequently, shifting intracellular c-di-GMP concentrations does not influence the activity of P*exsCEBA* when the promoter is inactivated by AmrZ. Interestingly, AmrZ regulates T3SS gene expression in an expression level-dependent manner. The basal expression level of AmrZ under growth conditions used in this study did not influence T3SS gene expression. Therefore, the reduced expression of *amrZ* in the Δ*fleR* mutant did not influence T3SS gene expression, which explains why T3SS gene expression was not altered by the deletion of *fleR* in the Δ*fleQ* mutant.

FleS and FleR were known to form a TCS and showed equal contributions to the regulation of multiple phenotypes. It was also found that FleS and FleR regulate swimming motility in *P. aeruginosa* with different patterns. Loss of *fleR* almost abolished the swimming motility whereas loss of *fleS* slightly inhibited swimming.[36] Similarly, we showed that only FleR is involved in the repression of T3SS gene expression. Regulation of T3SS by FleR was presumably independent of its phosphorylated status, since T3SS gene expression was repressed by FleR as well as its variants carrying mutations at aspartate residues to prevent the reception of phosphoryl groups, no matter in the WT strain or the Δ*fleS* mutant. FleR contains REC, AAA, and HTH domains. In the present study, we further demonstrated that the entire DNA-binding HTH domain of FleR is dispensable for the regulation of T3SS gene expression. All these data indicated that FleR regulates some physiological processes, such as T3SS gene expression, as an atypical TCS response regulator. Due to the presence of an AAA domain, FleR is also considered to be an enhancer-binding protein (EBP) in addition to being a response regulator. Thus, FleR may interact with RpoN (σ^{54}) to indirectly modulate downstream genes involved in c-di-GMP biosynthesis and degradation.[52] Future explorations on this speculation will be interesting and expected, which will advance our understanding on the regulatory mechanism of the atypical response regulator FleR.

Ca^{2+} is a well-established signaling molecule in eukaryotes and elevated levels of Ca^{2+} in various body fluids are observed in patients with cystic fibrosis.[53,54] In *P. aeruginosa*, Ca^{2+} is known to induce the acute-to-chronic virulence switch, and LadS is a known Ca^{2+}-responsive kinase that activates the switch process by repressing genes involved in acute virulence including T3SS genes through the Gac/Rsm pathway.[28] In this

study, we further demonstrated that FleR represses T3SS gene expression under Ca^{2+}-rich conditions. Given that FleR plays a critical role in biofilm formation which is a hallmark of chronic infection, the identification of its novel function to inactivate the acute virulence factor T3SS in response to Ca^{2+} elevation in the present study provides new insights into the adaptive mechanism of the acute-to-chronic virulence switch in *P. aeruginosa*.

Limitations of the study

Hinted by the structure of FleR-REC which showed the coordination of Ca^{2+} in the active site of FleR-REC, we demonstrated that FleR represses T3SS gene expression under the Ca^{2+}-rich condition. However, whether FleR directly responds to Ca^{2+} *in vivo* and key residues that interact with Ca^{2+} are not verified. In addition, we showed the regulation of T3SS gene expression by FleR through the c-di-GMP-mediated pathway in this study, but further investigations are still required to understand how FleR controls the intracellular content of c-di-GMP and how c-di-GMP and its effector FleQ controls T3SS gene expression.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109690.

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AUTHOR CONTRIBUTIONS

J.H., L.Z., and Ze.X. designed the research; J.H., Zi.X., and T.Z. performed experiments; J.H. and Ze.X. analyzed the data; J.H. drafted the manuscript; L.Z. and Ze.X. acquired fundings, supervised research, and revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-ExsA of *P. aeruginosa*	Lab collection	N/A
Mouse monoclonal anti-ExoS of *P. aeruginosa*	Lab collection	N/A
Mouse monoclonal anti-PcrV of *P. aeruginosa*	Lab collection	N/A
HRP, Goat Anti-Mouse IgG	Abbkine	RRID: AB_2728771
HRP, Goat Anti-Rabbit IgG	Abbkine	RRID: AB_2876889
Recombinant Anti-RNA polymerase beta antibody	Abcam	RRID: AB_3076167
Bacterial strains		
See Table S1	This Paper	N/A
Chemicals, peptides, and recombinant proteins		
BamHI-HF	New England Biolabs	R3136V
HindIII-HF	New England Biolabs	R0104V
Fetal Bovine Serum (FBS)	Gibco	REF# 10099141C
Dulbecco's Modified Eagle Medium (DMEM)	Gibco	REF# C11995500BT
Phanta Max Super-Fidelity DNA Polymerase	Vazyme	P505
Critical commercial assays		
ChamQ Universal SYBR qPCR master mix	Vazyme	Q711-02
2 × T5 Super PCR mix	Tsingke	Cat# TSE005
ClonExpress Ultra One Step Cloning Kit	Vazyme	C116-01
CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit	Promege	REF# G1780
Eastep® Super Total RNA Extraction Kit	Promege	REF# LS1040
EasyPure Bacteria Genomic DNA Kit	TransGen	Code# EE161-01
Enhanced ECL Chemiluminescence Kit (Ready-to-use)	Vazyme	E411-04
HiScript III 1st Strand cDNA Synthesis Kit (with gDNA wiper)	Vazyme	R312-01
TIANprep Mini Plasmid Kit	Tiangen	Cat# DP109-03
Universal DNA Purification Kit	Tiangen	Cat# DP214-03
Experimental models: Cell lines		
Human lung epithelial A549 cell lines	Lab collection	N/A
Oligonucleotides		
See Table S2	This Paper	N/A
Recombinant DNA		
See Table S1	This Paper	N/A
Software and algorithms		
ImageJ	National Institutes of Health (NIH)	https://imagej.nih.gov
GraphPad Prism 8	GraphPad Software	https://www.graphpad.com

RESOURCE AVAILABILITY

Lead contact

Further information and requests should be directed to the lead contact, Zeling Xu (zelingxu@scau.edu.cn).

Materials availability

This study did not generate new unique reagents. Plasmids and bacterial strains used in this study are available on request after completion of a Materials Transfer Agreement.

Data and code availability

- All data reported in this study will be shared by the lead contact upon request.
- This study does not report original code.
- Any additional information required to reanalyze the data reported in this study is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Bacterial strains

Bacterial strains used in this study are listed in Table S1. Bacterial strains were routinely cultured in LB medium (10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl) at 37°C with 200-rpm agitation. When necessary, antibiotics were added as follows: gentamicin at 50 μg/ml and tetracycline at 60 μg/ml for *P. aeruginosa*; gentamicin at 50 μg/ml, tetracycline at 10 μg/ml, ampicillin at 100 μg/ml and kanamycin at 50 μg/ml for *E. coli*.

A549 cell line

The A549 cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, United States) containing 10% fetal bovine serum (FBS) (Gibco, United States) at 37°C with 5% CO_2 and 95% humidity.

METHOD DETAILS

Construction of gene deletion mutants and gene expression plasmids

In-frame gene deletions of *P. aeruginosa* were performed according to the previously described protocol.[33] Briefly, ~500-bp upstream and downstream sequences of the target gene were amplified and cloned into the suicide vector pK18mobsacB. The resulting vector was introduced into *P. aeruginosa* by tri-parental mating with the helper plasmid pRK2013. Mutants were selected by the loss of sucrose susceptibility on LB agar plates containing 10% sucrose and verified by PCR and sequencing. For chromosomal gene complementation, the ORFs of target genes as well as their native promoters were first amplified by PCR using the genomic DNA of PAO1 as template and then cloned into the plasmid of pUC18T-mini-Tn7T-Gm. The constructed plasmids together with another plasmid pTNS2 plasmid were co-introduced into PAO1 strains by electroporation. Chromosomal integration of complemented genes was verified by PCR and sequencing. For *in trans* gene overexpression, the ORFs of target genes were amplified from the PAO1 genome and then ligated into pBBR1-MCS5 or pUCP18. The constructed plasmids were introduced into PAO1 strains by triparental mating. Bacterial cells containing the desired plasmids were selected and verified by PCR and sequencing.

Promoter activity assays

Promoter activity was detected by measuring β-galactosidase activity or GFP fluorescence. Promoters of target genes were first amplified and ligated to mini-CTX-*lacZ* or pPROBE-NT-*gfp*. Constructs were transformed into *P. aeruginosa* strains and verified by colony PCR. For β-galactosidase activity assay, 1 ml bacterial cells were harvested at OD_{600} of 1.0 by centrifugation at 13,000 rpm for 2 min and then resuspended in 1 ml Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol). After 100 μl bacterial suspension was diluted with 500 μl Z buffer, 20 μl chloroform and 20 μl 0.1% SDS were added, and bacterial cells were lysed by vortex thoroughly. 100 μl 4% (w/v) o-nitrophenyl-β-D-galactoside (ONPG) was then added into the suspension and the suspension was incubated for 10 min. 250 ml 1 M Na_2CO_3 was added to stop the reaction. The promoter activity in Miller Units was calculated and relative promoter activity was presented. For GFP fluorescence assay, overnight bacterial cultures were diluted to OD_{600} of 0.02 and then sub-cultured in 96-well plates containing Volger-Bonner minimal medium (0.2 g/L $MgSO_4 \cdot 7H_2O$, 2 g/L citric acid, 3.5 g/L $NaNH_4HPO_4 \cdot 7H_2O$ and 10 g/L K_2HPO_4). Cell densities and fluorescence were measured every 2 h using a microplate reader.

RNA extraction and quantitative real-time PCR

Overnight bacterial cultures were sub-cultured into fresh LB broth and incubated with 200 rpm agitation till OD_{600} of 1.0. 1 ml bacterial culture was harvested and centrifugated at 13,000 rpm for 1 min. The Eastep super total RNA extraction kit (Promega, United States) was used for RNA purification. RNA concentration was measured, and 1 μg RNA was used to generate cDNA using the HiScript III 1st Strand cDNA Synthesis Kit (with gDNase) (Vazyme, China). Quantitative real-time PCR was performed in the QuantStudio 6 real-time detection system (Applied Biosystems, United States) using the ChamQ Universal SYBR qPCR master mix (Vazyme, China). The housekeeping 50S ribosomal protein gene *rplU* was used as the internal control.[55] Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.[56] The result was displayed as the mean of three biological repeats and four technical repeats.

Western blot analysis

All strains were cultured to OD_{600} of 1.0 and 10 ml of them were harvested by centrifugation at 13,000 rpm for 2 min. Cell pellets were resuspended with 200 μl RIPA lysis buffer (Biosharp, China) and incubated at 20°C for 30 min. After centrifugation, 50 μl SDS-PAGE sample buffer

was added into the suspension and the suspension was heated at 95°C for 10 min. Total proteins were separated using SmartPAGE™ 4-20% Precast Protein Gel (Smart-Lifescience, China) prior to be transferred to a PVDF membrane (Millipore, United States) by electroblotting. Membranes were blocked using PBST (PBS supplemented with 1% (v/v) Tween-20) containing 5% skim milk for 1 h at room temperature and then hybridized with primary antibodies against ExoS, PcrV or ExsA. Membranes were then probed with HRP-conjugated goat anti-mouse antibody (Abbkine, United States). Proteins were detected using the ECL kit and visualized with a cooled CCD camera (Tanon, China). Each experiment was repeated at least twice, and a representative result was selected and shown.

Quantification of c-di-GMP

Quantification of c-di-GMP was performed as described previously with minor modifications.[57] Bacteria were cultured in LB medium to OD_{600} of 1.0 and cells were collected by centrifugation. Cell pellets were resuspended in 1 ml Milli-Q water and then 94.2 μl 70% (v/v) perchloric acid was added and vortex thoroughly. The mixture was incubated on ice for 30 min. After centrifugation at 15,000 rpm for 10 min, supernatants were transferred to new tubes and 2.5 M $KHCO_3$ was added. After centrifugation at 15,000 rpm for 10 min, supernatants were subjected to LC-MS analysis or stored at -80°C. LC-MS was carried out using an Q Exactive Focus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, United States) with a 2.1×100-mm HSS T3 1.8 μm column (Waters, United States). The m/z 691>248 transition was used for quantification of c-di-GMP.

Protein purification

The *E. coli* BL21(DE3) strain carrying the pET-*amrZ* vector was cultured in 1 L LB medium to OD_{600} of 0.5-0.8. 0.5 mM IPTG was added, and the cell culture was incubated at 16°C for protein expression. Cell pellets were harvested by centrifugation and resuspended in 100 ml lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole). Cells were lysed by sonication. After centrifugation at 10,000 rpm for 30 min, the supernatant was filtered with a 0.22 μm filter before loaded into the Ni NTA beads column (Smart-Lifescience, China). The AmrZ protein was collected with elution buffer supplemented with 200 mM imidazole. Purified AmrZ protein was stored at -80°C.

Electrophoretic mobility shift assay (EMSA)

Ethidium bromide (EB)-stained EMSA was performed as previously described.[58] DNA probes were amplified by PCR from the PAO1 genome. 50 ng DNA probes were incubated with the purified AmrZ protein (200 ng, 400 ng, 600 ng, 800 ng) in EMSA binding buffer for 20 min at 37°C. The 20-μl reaction mixture was subjected to 6% native PAGE at 120 V for 90 min. The gel was incubated in the 0.5 × TBE buffer which contained EB at room temperature for 5 min and then visualized under UV illumination.

Cytotoxicity assay

Bacterial cytotoxicity was determined by evaluating the activity of lactate dehydrogenase (LDH) released from A549 cells after the infection by *P. aeruginosa*.[11] 1.5×10^4 A549 cells were seeded into a 96-well plate and cultured overnight in DMEM (Gibco, United States) containing 10% FBS (Gibco, United States) at 37°C with 5% CO_2 and 95% humidity. *P. aeruginosa* cells were cultured in LB medium until OD_{600} of 1.0. 1 ml bacterial cells were centrifuged and resuspended in 1 ml DMEM with 1% FBS which maintains the osmotic pressure and reduces proliferation of the A549 cells during infection. Before infection, A549 cells were washed twice with 100 μl PBS buffer. Then A549 cells were infected with bacteria at a multiplicity of infection (MOI) of 50 for 4 h. Cytotoxicity was measured by CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega, United States) following the manufacturer's instructions.

Pull-down assay

The open reading frames of *fleQ* and *rsmA* genes were amplified from the PAO1 genome and cloned into vectors pGEX-6p-1 and pET-28a, respectively. The constructs were transformed into *E. coli* BL21(DE3) for the expression of GST-tagged FleQ (FleQ-GST) and His-tagged RsmA (RsmA-His) proteins. Pull-down assay was performed as previously described.[11] Briefly, Ni NTA beads 6FF was firstly mixed with the whole-cell lysate of BL21(DE3) which expresses RsmA-His and incubated at 4°C for 2 h. After unbound proteins were washed off, whole-cell lysate of BL21(DE3) which expresses FleQ-GST was added and incubated at 4°C for 16 h. After washing, proteins were eluted and mixed with loading buffer. Samples were heated at 95°C for 5 min and supernatants were collected for SDS-PAGE and western blot analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical details of the experiments, where applicable, can be found in figure legends.