



Elongation factor P modulates *Acinetobacter baumannii* physiology and virulence as a cyclic dimeric guanosine monophosphate effector

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Cyclic diguanosine monophosphate (c-di-GMP) is widely used by bacteria to control biological functions in response to diverse signals or cues. A previous study showed that potential c-di-GMP metabolic enzymes play a role in the regulation of biofilm formation and motility in *Acinetobacter baumannii*. However, it was unclear whether and how *A. baumannii* cells use c-di-GMP signaling to modulate biological functions. Here, we report that c-di-GMP is an important intracellular signal in the modulation of biofilm formation, motility, and virulence in *A. baumannii*. The intracellular level of c-di-GMP is principally controlled by the diguanylate cyclases (DGCs) A1S_1695, A1S_2506, and A1S_3296 and the phosphodiesterase (PDE) A1S_1254. Intriguingly, we revealed that A1S_2419 (an elongation factor P [EF-P]), is a novel c-di-GMP effector in *A. baumannii*. Response to a c-di-GMP signal boosted A1S_2419 activity to rescue ribosomes from stalling during synthesis of proteins containing consecutive prolines and thus regulate *A. baumannii* physiology and pathogenesis. Our study presents a unique and widely conserved effector that controls bacterial physiology and virulence by sensing the second messenger c-di-GMP.

Acinetobacter baumannii | c-di-GMP | elongation factor P | physiology | pathogenicity

The second messenger bis-(3'-5')-cyclic guanosine monophosphate (c-di-GMP) controls the bacterial biofilm and motility switch and many other biological functions, including quorum sensing signal production and virulence (1–4). It is enzymatically synthesized from two molecules of guanosine-5'-triphosphate (GTP) by diguanylate cyclases (DGCs) harboring GGDEF domains and degraded to the linear dinucleotide pGpG or to guanosine monophosphate (GMP) by c-di-GMP-specific phosphodiesterases (PDEs) containing EAL or HD-GYP domains (5, 6). Many bacterial species contain multiple proteins involved in c-di-GMP synthesis and degradation, indicating the importance and diverse roles of the c-di-GMP signaling system in bacterial physiology (7, 8).

The c-di-GMP metabolic enzymes are readily predictable because of the characteristic GGDEF, EAL, and HD-GYP domains. However, it is challenging to decipher how c-di-GMP could act on target genes, because this signal has diverse receptors or effector proteins with unconserved binding sites and motifs (5). A few c-di-GMP receptor families, including PilZ domain receptors, inhibitory site receptors, and EAL domain receptors, can be predicted on the basis of primary sequences (5). There are also some unpredictable c-di-GMP receptors that have binding sites without defined consensus motifs that include various kinds of transcriptional regulators and proteins with diverse and unrelated functions (3–5, 9–14). Recently, the cis-2-dodecenoic acid receptor RpfR was found to be a c-di-GMP sensor, and binding of c-di-GMP by RpfR decreased the affinity of the RpfR-GtrR complex to target promoter DNA (15, 16). In addition, members of a particular class of riboswitches were also found to bind to c-di-GMP (17, 18).

Proline contains a unique constrained ring structure that makes it a poor peptide bond donor and acceptor (19). Polyproline stretches cause ribosome pausing and influence the translation elongation rate (20). In addition to polyproline-containing proteins, specific subsets of proteins containing diprolyl motifs (XPP/PPX) also cause ribosome pausing (21). Elongation factor P (EF-P) binds the stalled ribosomes and facilitates peptide bond formation between prolines by forcing the prolines to adopt an alternative conformation (22, 23).

Here, we found that A1S_2419, an EF-P, controls biological functions, including biofilm formation, motility, and virulence, in the human pathogen *Acinetobacter baumannii*. A1S_2419 controls the translation efficiency of target proteins with consecutive prolines, and the binding of c-di-GMP enhances the function of A1S_2419 to promote translation efficiency. Thus, A1S_2419 serves as a unique sensor and effector of c-di-GMP. Taken together, our findings have unveiled a novel effector of c-di-GMP, which enables

Significance

Many bacteria use the second messenger cyclic diguanosine monophosphate (c-di-GMP) to control diverse biological functions. Here, we show that A1S_2419, an elongation factor P (EF-P) from the human pathogenic bacterium *Acinetobacter baumannii*, is a c-di-GMP effector protein. It controls the translation efficiency of the target proteins with consecutive prolines. Intriguingly, A1S_2419 modulates biofilm formation, motility, and virulence in *A. baumannii* in a c-di-GMP-dependent manner. This article describes a novel c-di-GMP signaling mechanism with which a pathogen uses EF-P to integrate the second messenger signal turnover and translational regulation of target genes.

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The authors declare no competing interest.

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integration of the intracellular c-di-GMP signal into translational machinery to modulate bacterial physiology and virulence.

Results

c-di-GMP Is Involved in the Regulation of Biological Functions and Virulence in *A. baumannii*. It was previously revealed that potential c-di-GMP metabolic enzymes are involved in the regulation of biofilm formation and motility in *A. baumannii* (24). To verify the existence of intracellular c-di-GMP, the collected extract of *A. baumannii* American Type Culture Collection (ATCC) 17978 cells was analyzed by using liquid chromatography coupled with tandem mass spectrometry. As shown by triple-quadrupole mass spectrometry (SI Appendix, Fig. S1A), the simultaneous appearance of three characteristic mass transitions (m/z 691/152, m/z 691/248, and m/z 691/540) verified the existence of c-di-GMP in *A. baumannii* cells.

To further confirm that changes in the intracellular level of c-di-GMP could affect the cellular functions of *A. baumannii*, we expressed in trans the *rocR* and *wspR* genes from *Pseudomonas aeruginosa* that encode a well-characterized c-di-GMP phosphodiesterase and synthase, respectively, in the wild-type strain of *A. baumannii*. As expected, the expression of the c-di-GMP degradation protein RocR caused a decrease in the intracellular c-di-GMP level and resulted in decreased biofilm formation capacity but enhanced the ability to move on a semisolid surface termed “surface motility” (25) (SI Appendix, Fig. S1 B–D). In contrast, overexpression of *wspR* increased the intracellular concentration of c-di-GMP in *A. baumannii* and promoted biofilm formation but reduced motility (SI Appendix, Fig. S1 B–D). *A. baumannii* usually causes lung infections (26). The human lung adenocarcinoma cell line A549 was used as an in vitro model to test the effect of intracellular c-di-GMP levels on the cytotoxicity of *A. baumannii*. It was revealed that in trans expression of *rocR* in the wild-type strain decreased cytotoxicity by 33%, whereas overexpression of *wspR* increased the cytotoxicity of *A. baumannii* by 121% (SI Appendix, Fig. S1E). These results suggest that c-di-GMP signaling regulates the important biological functions and pathogenicity of *A. baumannii*.

c-di-GMP Metabolic Enzymes Affect Biological Functions through c-di-GMP. There are 11 potential c-di-GMP metabolic enzyme-encoding genes that have been identified in the genome of *A. baumannii* ATCC 17978 (24). Six genes encoding the GGDEF domain were located at the *AIS_0751*, *AIS_1067*, *AIS_1695*, *AIS_2506*, *AIS_2986*, and *AIS_3296* loci. Two genes encoding the EAL domain were located at the *AIS_1254* and *AIS_2422* loci, and three genes encoding a GGDEF and an EAL domain were located at the *AIS_0546*, *AIS_1949*, and *AIS_2337* loci. We found a new GGDEF domain-encoding gene at the *AIS_3345* loci by using the Basic Local Alignment Search Tool (BLAST; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) algorithm (SI Appendix, Table S1), but we did not find any gene encoding HD-GYP domain in the genome of *A. baumannii* ATCC 17978.

We then investigated the possible relationship between the potential c-di-GMP metabolic enzymes and intracellular levels of c-di-GMP in *A. baumannii*. Considering the existence of compensatory mechanisms and functional redundancy in bacteria, we constructed a DGC-null mutant of *A. baumannii* 17978 by deleting all GGDEF domain-encoding genes. Starting with deletion of *AIS_2506*, we then sequentially deleted the other GGDEF domain-encoding genes, including *AIS_0751*, *AIS_1067*, *AIS_1695*, *AIS_2986*, *AIS_3296*, and *AIS_3345*, and the GGDEF domain-encoding regions of *AIS_0546*, *AIS_1949*, and

AIS_2337 in *A. baumannii* ATCC 17978, generating the Δ 10DGC deletion mutant strain. The Δ 10DGC deletion mutant strain exhibited stronger motility and an obvious reduction in both biofilm formation and cytotoxicity compared with the wild-type strain (Fig. 1 A–C). Consistent with this, the intracellular c-di-GMP level in the Δ 10DGC mutant was almost undetectable (Fig. 1D).

An in-frame deletion mutant strain was also constructed by deleting EAL domain-encoding gene *AIS_1254* and the EAL domain-encoding regions of *AIS_2337*, *AIS_1949*, and *AIS_0546*, but not that of *AIS_2422*. *AIS_2422* did not exhibit PDE activity and c-di-GMP binding ability in vitro, and in-frame deletion of it did not affect the intracellular level of c-di-GMP (SI Appendix, Fig. S2). In addition, the catalytic site and loop 6 motif of the *AIS_2422* EAL domain are alanine residue (Ala⁶⁷⁰) and RDFASSMYS⁶²¹, respectively, which deviate from glutamine residue (Glu³⁵²) and DDFGAGYSS³⁰³ of a typical EAL domain-containing protein such as RocR (27, 28) (SI Appendix, Table S1). The generated Δ 4PDE mutant strain showed impaired motility and clearly enhanced biofilm formation and cytotoxicity (Fig. 1 A–C). In contrast to the intracellular level of c-di-GMP in

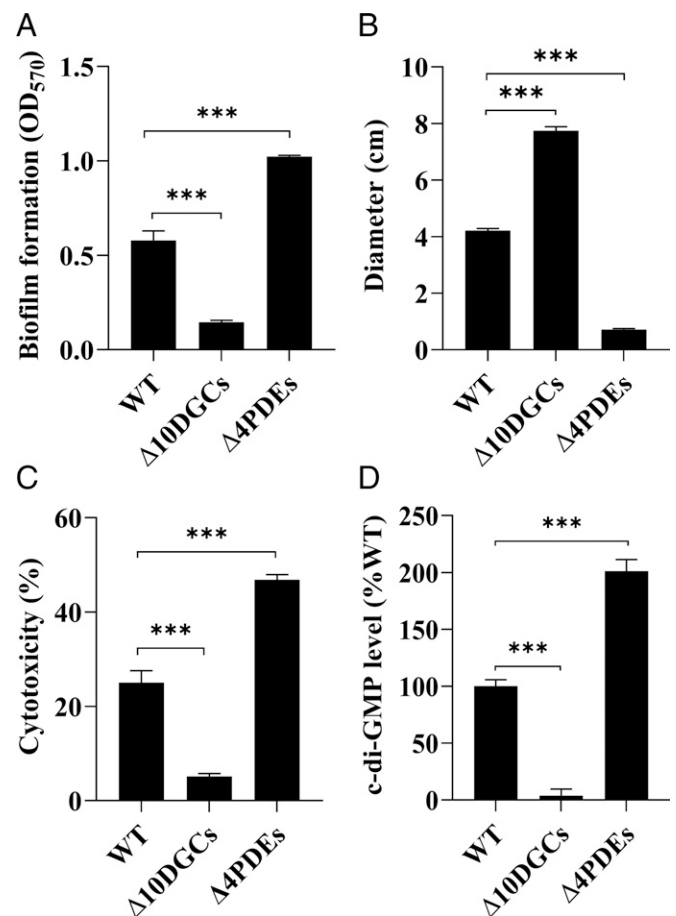


Fig. 1. Phenotypes of multiple c-di-GMP metabolic enzyme-encoding gene deletion mutants. The Δ 10DGC strain was constructed by deleting *AIS_0751*, *AIS_1067*, *AIS_1695*, *AIS_2506*, *AIS_2986*, *AIS_3296*, and *AIS_3345* and the GGDEF domain-encoding regions of *AIS_0546*, *AIS_1949*, and *AIS_2337*. The Δ 4PDE strain was generated by deleting *AIS_1254*, and the EAL domain-encoding regions of *AIS_0546*, *AIS_1949*, and *AIS_2337*. The biofilm formation (A), surface-associated motility (B), cytotoxicity (C), and intracellular c-di-GMP level (D) of DGC- and PDE-null mutants were measured. For ease of comparison, the c-di-GMP level of each mutant was normalized to that of the wild-type (WT) strain. The results were analyzed using one-way ANOVA for at least three independent experiments. *** $P < 0.001$. Error bars indicate the standard deviations (SD).

the $\Delta 10\text{DGC}$ mutant strain, the level of c-di-GMP in the $\Delta 4\text{PDE}$ mutant strain was increased to 201% of that in the wild-type strain (Fig. 1D).

Major DGCs and PDEs in *A. baumannii*. To further study the enzymatic activity and roles of these c-di-GMP metabolic enzymes in *A. baumannii*, we complemented the $\Delta 10\text{DGC}$ and $\Delta 4\text{PDE}$ mutant strains with each single c-di-GMP metabolic enzyme. As shown in *SI Appendix, Fig. S3A*, in trans expression of A1S_1695, A1S_2506, and A1S_3296 increased the biofilm formation of the $\Delta 10\text{DGC}$ mutant strains by 228%, 443%, and 352%, respectively. The motility of the $\Delta 10\text{DGC}$ mutant strains was reduced by 35%, 44%, and 31% with overexpression of A1S_1695, A1S_2506, and A1S_3296, respectively (*SI Appendix, Fig. S3B*). The analysis results for the model human cell line showed that in trans expression of A1S_1695, A1S_2506, and A1S_3296 partially restored the virulence of the $\Delta 10\text{DGC}$ mutant strains to the wild-type level (*SI Appendix, Fig. S3C*). We continued to test the potential role of the PDEs and found that in trans expression of A1S_1254 in the $\Delta 4\text{PDE}$ mutant strain increased the motility of this mutant strain by 357% (*SI Appendix, Fig. S3E*) and restored biofilm formation and cytotoxicity to the wild-type level (*SI Appendix, Fig. S3 D and F*).

The in vitro DGC or PDE activity of A1S_1695, A1S_2506, A1S_3296, and A1S_1254 was also investigated. More than 86% of GTP was converted to c-di-GMP by the A1S_2506 protein within 5 min (*SI Appendix, Fig. S4 A–C*), while the amount of GTP converted by A1S_1695 and A1S_3296 was only ~70% within 2 h (*SI Appendix, Figs. S4 D–F and G–I*). We observed that A1S_1254 degraded almost all of the c-di-GMP to GMP rather than pGpG within 5 min (*SI Appendix, Fig. S4 J–L*). Because A1S_0546, A1S_1949, and A1S_2337 all contain a GGDEF-EAL tandem, their DGC and PDE activities were also investigated. Both of the GGDEF domains of A1S_0546 and A1S_1949 showed a weak activity for converting GTP to c-di-GMP, while the in vitro DGC activity of A1S_2337 GGDEF domain was not observed (*SI Appendix, Fig. S5 A–G*). Similar to A1S_1254, the EAL domain of A1S_2337 degraded c-di-GMP to GMP, while the EAL domains of A1S_0546 and A1S_1949 hydrolyzed c-di-GMP to pGpG and further to GMP (*SI Appendix, Fig. S5 H–N*). It was also previously observed that some proteins containing the EAL domain hydrolyzed c-di-GMP to pGpG, and then pGpG was further degraded to GMP (28, 29). In this study, we observed that after incubation with the EAL domains of A1S_0546, A1S_1254, A1S_1949, and A1S_2337, c-di-GMP was finally hydrolyzed to GMP (*SI Appendix, Fig. S5*).

The effect on the c-di-GMP concentration of deleting each individual gene was also investigated. Interestingly, only the $\Delta A1S_1254$ mutant showed a 40% increase, and the $\Delta A1S_1695$, $\Delta A1S_2506$, and $\Delta A1S_3296$ mutants exhibited 16%, 20%, and 18% decreases, respectively, in the level of c-di-GMP compared with that of the wild-type strain (*SI Appendix, Fig. S6A*). These results agree well with the results of the phenotypic analysis. Consistent with the elevated intracellular c-di-GMP concentration, the ability of the $\Delta A1S_1254$ mutant to form biofilms increased by 38%, but the motility was decreased by 25% (*SI Appendix, Fig. S6 B and C*). In contrast, $\Delta A1S_1695$, $\Delta A1S_2506$, and $\Delta A1S_3296$ displayed 14%, 28%, and 15% decreases, respectively, in biofilm formation, but 16%, 43%, and 39% increases, respectively, in motility activity compared with the wild-type strain (*SI Appendix, Fig. S6 B and C*). Together, these results suggested that A1S_1695, A1S_2506, A1S_3296, and A1S_1254

are the main c-di-GMP turnover proteins in *A. baumannii* ATCC 17978 under the investigated experimental conditions.

To comprehensively determine the regulatory roles of c-di-GMP in the transcriptome of *A. baumannii* ATCC 17978, we analyzed and compared the gene transcription profiles of the wild-type strain and the $\Delta A1S_1254$ mutant strain by using RNA sequencing (RNA-seq). Differential gene expression analysis showed that 72 genes were upregulated and 66 genes were downregulated (\log_2 fold-change ≥ 1.5) in the $\Delta A1S_1254$ mutant strain compared with that in the wild-type strain (*SI Appendix, Fig. S7 and Table S2*). These genes were associated with a diverse range of biological functions, including cell motility, DNA replication, transport, transcription, membrane components, and signal transduction (*SI Appendix, Fig. S7A and Table S2*). qRT-PCR analysis of select genes confirmed the RNA-seq results (*SI Appendix, Fig. S7B*). These genes include the *csu pilus* (*A1S_2213* to *A1S_2218*) and outer membrane protein A (*A1S_2840*) genes, which were shown to be involved in biofilm formation in *A. baumannii* (30, 31).

EF-P Rescues the Phenotypes of $\Delta 10\text{DGCs}$. Because c-di-GMP plays a role in regulating the important biological functions and virulence of *A. baumannii*, we next searched for homologs of c-di-GMP effectors in the genome of *A. baumannii* ATCC 17978 to identify the potential downstream component of the c-di-GMP signaling system by using the BLAST program. We found 13 potential homologs of c-di-GMP effectors sharing 24.42–52.63% of their identity with the identified c-di-GMP effectors or receptors (*SI Appendix, Table S3*). We expressed these homologs in trans in the $\Delta 10\text{DGC}$ mutant and measured the biofilm formation and motility of these overexpression strains. The results showed that in trans expression of A1S_2421, which contains a PilZ domain analyzed by the Pfam database (pfam.xfam.org), partially rescued the biofilm formation defect and reduced the motility of $\Delta 10\text{DGCs}$ (*SI Appendix, Fig. S8*). Interestingly, in trans expression of the neighboring gene of *A1S_2421* (*A1S_2419*) also showed a similar effect in restoring all the tested phenotypes of $\Delta 10\text{DGCs}$, including biofilm formation, motility, and cytotoxicity, which are all regulated by the c-di-GMP signaling system (Fig. 2). Because the PilZ domain is widely recognized as a c-di-GMP effector, we then focused our attention on A1S_2419.

A1S_2419 is an *efp* encoding EF-P (Fig. 2 A and B), which recognizes and binds to the E-site region of ribosomes when it translates proteins containing consecutive prolines (32). Previous studies have shown that EF-P plays an important role in bacterial motility, fitness, and pathogenicity (33–36). Proteome analysis revealed that *A. baumannii* ATCC 17978 contained a total of 1,367 XPPX motifs, and approximately 60 proteins contained 3 or more consecutive prolines (*SI Appendix, Table S4*), including BfmS and CsuD. BfmS is the sensor kinase of the two-component system BfmSR, which controls the production of outer membrane vesicles (37), and CsuD is an important constituent of the *csu pilus* (31). Both of those proteins have been identified as being involved in biofilm formation, motility, and virulence in *A. baumannii* (31, 37–39). Because there is no information on the relationship between EF-P and c-di-GMP, we continued to study the role of A1S_2419 in the c-di-GMP signaling pathways in *A. baumannii*.

EF-P Modulates c-di-GMP-Regulated Phenotypes. We then tried to prepare an *efp*-null mutant by inserting a gentamycin cassette into the gene or deleting the region encoding *efp* from the *A. baumannii* ATCC 17978 genome. However, we failed to obtain any mutant colonies. The results suggested that this

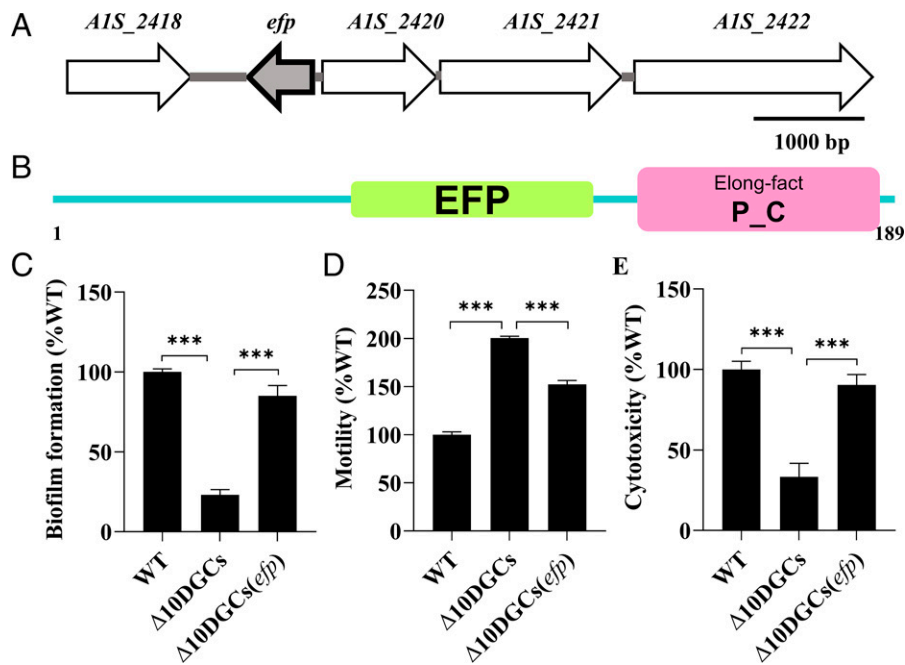


Fig. 2. EF-P complements the phenotypes of the $\Delta 10DGC$ mutant strain. Genomic organization of the region encoding EF-P in *A. baumannii* ATCC 17978 (A) and domain structure analysis of EF-P (B). EFP, elongation factor P oligonucleotide binding domain; Elong-fact P_C, elongation factor P, C-terminal. The domains were analyzed by using SMART. Overexpression of *efp* promoted the biofilm formation (C), inhibited the surface-associated motility (D), and increased the cytotoxicity (E) of the $\Delta 10DGC$ mutant. The results in C–E were analyzed using one-way ANOVA for at least three independent experiments. *** $P < 0.001$. WT, wild-type. Error bars indicate the standard deviations (SD).

gene may be essential for *A. baumannii*. To further confirm this possibility, *A. baumannii* cells were supplemented with a pME2 plasmid carrying the *efp* gene, which was expressed under the control of a vanillate-inducible promoter (3). In the presence of 1 mM vanillate, we successfully generated an in-frame *efp* deletion mutant ($\Delta efp/Pvan-efp$). The deletion mutant strain could not survive in the absence of vanillate (Fig. 3A), suggesting that *efp* is an essential gene for *A. baumannii*. The essentiality of EF-P is debated and may differ among organisms. For example, it is an essential gene for *Neisseria meningitidis* viability (40). However, in some other bacteria, including *Escherichia coli*, deletion of EF-P is not lethal, but it severely impairs bacterial growth (35, 36). As shown in Fig. 3B, the $\Delta efp/Pvan-efp$ mutant exhibited an extended lag phase when cultured at 37 °C with shaking, even in the presence of 1 mM vanillate. In addition, the cell density of $\Delta efp/Pvan-efp$ was significantly lower than that of the wild-type strain when the cells were cultured under stationary conditions (SI Appendix, Fig. S9). The results indicated that *efp* plays an important role in *A. baumannii* growth.

In the presence of 1 mM vanillate, the transcription level of *efp* in the $\Delta efp/Pvan-efp$ mutant was significantly lower than that in the wild-type strain (Fig. 3C). To test the impacts of *efp* on the yield of proteins containing consecutive prolines, we transformed a reporter plasmid containing enhanced green fluorescent protein (EGFP) or EGFP with four consecutive proline residues inserted at the N terminus (4P-EGFP) into the $\Delta efp/Pvan-efp$ strain; we then measured the fluorescence intensity of EGFP or 4P-EGFP when cells were grown to an OD₆₀₀ (optical density at 600 nm) of 1.0. As expected, the EGFP fluorescence in the $\Delta efp/Pvan-efp$ strain was similar to that in the wild-type strain, while the fluorescence of 4P-EGFP in the $\Delta efp/Pvan-efp$ strain was reduced about 10-fold compared with that in the wild-type strain (Fig. 3D), confirming the EF-P role of AIS_2419. We also observed that the biofilm formation of $\Delta efp/Pvan-efp$ was reduced by 77%, while its motility was increased by ~24% (Fig. 3 E and F) compared with

that of the wild-type strain. To test whether *efp* impacts the pathogenicity of *A. baumannii*, we investigated the wild-type strain and the $\Delta efp/Pvan-efp$ mutant in a cell line infection assay. As shown in Fig. 3G, the cytotoxicity of the $\Delta efp/Pvan-efp$ strain decreased to 45% of that of the wild-type *A. baumannii* strain. To verify that these phenotypic changes were due to deletion of the *efp* locus, we complemented $\Delta efp/Pvan-efp$ strain with pAb-MCS plasmid containing *efp* and its native promoter to generate the complemented strain $\Delta efp/Pvan-efp(efp)$. It was observed that complementation fully restored the phenotypes to levels comparable to those in the wild-type strain (Fig. 3 D and E). Together, these results showed that EF-P is an essential gene for *A. baumannii* and mediates c-di-GMP-regulated phenotypes.

EF-P Binds c-di-GMP. Since EF-P modulates c-di-GMP-regulated functions and since it complemented the phenotypes of $\Delta 10DGCs$, we first speculated that c-di-GMP may regulate *efp* transcription. However, the transcriptional levels of *efp* were similar among the wild-type, $\Delta 10DGC$, and $\Delta 4PDGE$ strains (Fig. 3C), and Western blotting assays showed that there is no difference among the EF-P levels in these strains (SI Appendix, Fig. S10). These results suggested that c-di-GMP may influence the activity of EF-P through ligand–protein interactions. Therefore, we then applied differential scanning fluorimetry (DSF) and microscale thermophoresis (MST) to test whether EF-P interacts with c-di-GMP. The DSF assay showed a marked shift in the melting curve of EF-P, suggesting that EF-P binds to c-di-GMP (Fig. 4 A and B). MST analysis revealed that this protein bound c-di-GMP with an estimated dissociation constant (KD) of $9.82 \pm 0.54 \mu M$ (Fig. 4C).

To further confirm the binding between EF-P of *A. baumannii* and c-di-GMP, we used isothermal titration calorimetry analysis, and the result showed that EF-P tightly bound to c-di-GMP with an estimated KD of $5.9 \mu M$ (Fig. 4D). c-di-GMP is a ubiquitous second messenger and EF-P is widely conserved in diverse bacteria, so we investigated whether EF-P proteins of other bacterial

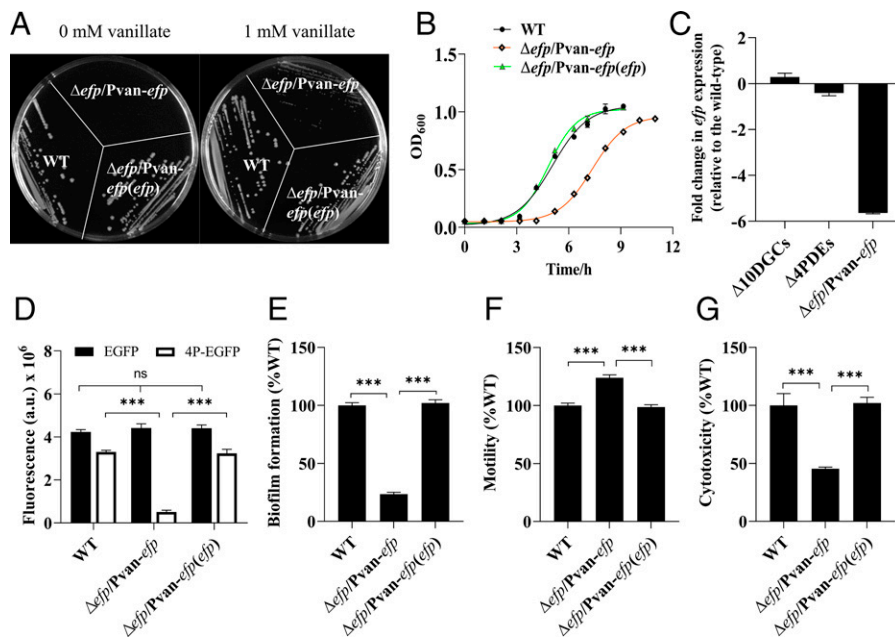


Fig. 3. Effects of EF-P on *A. baumannii* physiology. (A) Growth of the wild-type (WT), $\Delta efp/Pvan-efp$, and $\Delta efp/Pvan-efp$ complemented with *efp* under the control of its native promoter ($\Delta efp/Pvan-efp(efp)$) strains on the solid surface in the absence or presence of vanillate (1 mM). The plates were incubated for 12 h. The experiment was performed three times, and representative images from one experiment are shown. (B) The $\Delta efp/Pvan-efp$ mutant had an extended lag phase compared with the wild-type strain. Cells were grown in LB broth supplemented with vanillate (1 mM) with shaking. (C) qRT-PCR analysis of *efp* expression in the $\Delta 10DGC$, $\Delta 4PDE$, and $\Delta efp/Pvan-efp$ strains compared with that of the wild-type strain. (D) The total yield of EGFP with four proline residues at the N terminus (4P-GFP) was affected in the $\Delta efp/Pvan-efp$ mutant. Data were analyzed using two-way ANOVA for three independent experiments. The fluorescence was measured when cells were grown to $OD_{600} = 1.0$. a.u., arbitrary units. Biofilm formation (E), motility (F), and cytotoxicity (G) of the $\Delta efp/Pvan-efp$ mutant were measured in the presence of 1 mM vanillate. For ease of comparison, the phenotypes of the $\Delta efp/Pvan-efp$ mutant were normalized to those of the wild-type strain. The results in D–G are analyzed using one-way ANOVA for at least three independent experiments. *** $P < 0.001$. ns, not significant. Error bars indicate the standard deviations (SD).

species, including *E. coli*, *P. aeruginosa*, *Burkholderia cenocepacia*, and *A. baumannii*, bind to c-di-GMP. Intriguingly, we found that the EF-P proteins from *E. coli*, *P. aeruginosa*, and *B. cenocepacia* did not bind to c-di-GMP, but the EF-P of *A. baumannii* bound to c-di-GMP with an estimated KD of 10.7 μ M (SI Appendix, Fig. S11). We also tested some other compounds such as GMP, adenosine monophosphate (AMP), and cyclic adenosine monophosphate (cAMP) and found that EF-P did not bind them (SI Appendix, Fig. S12), suggesting that the binding between EF-P and c-di-GMP is specific.

We then continued our attempt to identify the binding sites of c-di-GMP in the EF-P of *A. baumannii*. Autodocking analysis revealed five amino acid residues at positions Lys47(K47), Glu69(E69), Asp102(D102), Val127(V127), and Asn128(N128) that might be critical for the interaction between EF-P and

c-di-GMP (SI Appendix, Fig. S13A). We then generated five single-point mutants (EF-P^{K47A}, EF-P^{E69A}, EF-P^{D102A}, EF-P^{V127A}, and EF-P^{N128A}) (SI Appendix, Fig. S13B). MST analysis showed that mutations at K47 and E69 remarkably weakened the binding between EF-P and c-di-GMP (SI Appendix, Fig. S13 F and G).

c-di-GMP Enhances the Modulatory Effect of EF-P on the Translation of Proteins Containing Consecutive Prolines. We then investigated the influence of c-di-GMP on the function of EF-P to control the translation efficiency of the target proteins with consecutive prolines. As shown in Fig. 5A, there was no difference in the fluorescence of EGFP among the wild-type, $\Delta 10DGC$, and $\Delta 4PDE$ mutant strains. The 4P-EGFP level in the $\Delta 10DGC$ mutant and $\Delta 4PDE$ mutant was 11% and 130%

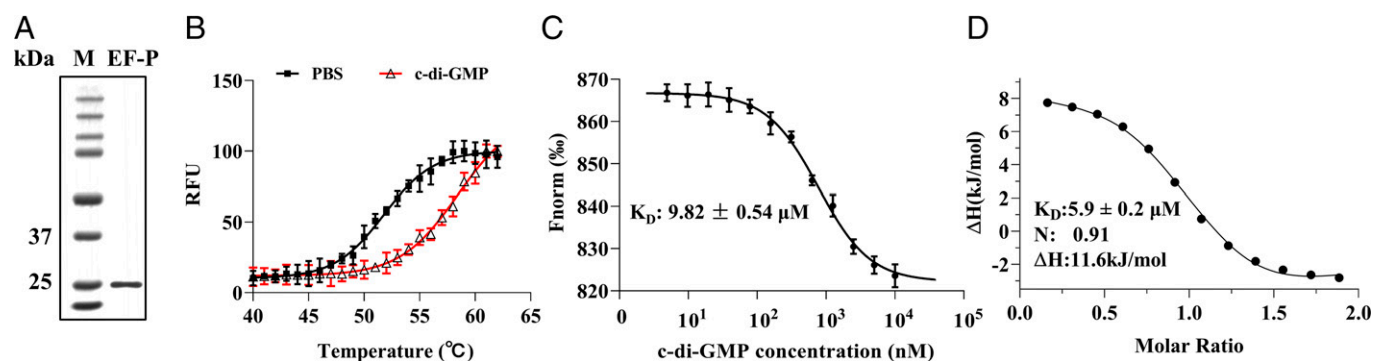


Fig. 4. c-di-GMP binds to EF-P (A1S_2419). (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified EF-P protein. M, protein marker. (B) Protein melting curves of EF-P in the absence or presence of c-di-GMP (500 μ M). The maximal fluorescence was normalized by 100%. PBS, phosphate-buffered saline; RFU, relative fluorescence units. Error bars indicate the standard deviations (SD). (C) MST analysis of the interaction of c-di-GMP and EF-P. Fnorm (%) indicates the fluorescence time trace changes in the MST response. Error bars indicate the standard deviations (SD). (D) Isothermal titration calorimetry (ITC) analysis of the molecular interaction between c-di-GMP and EF-P of *A. baumannii*. The results in B and C are the mean \pm SD of at least three independent experiments. N, binding stoichiometry; H, enthalpy.

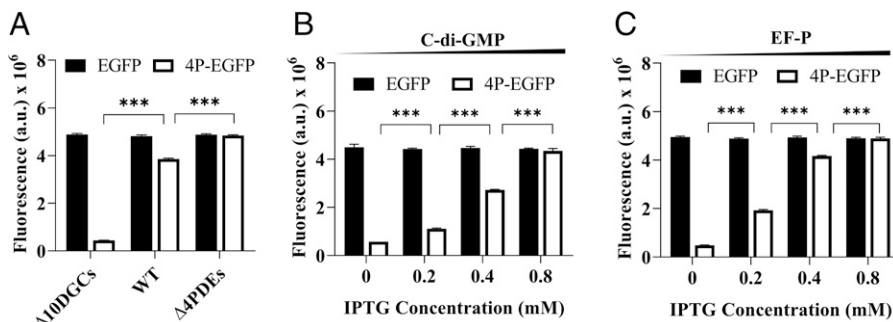


Fig. 5. *c*-di-GMP influences the translation of polyproline motif-containing proteins through EF-P. (A) The fluorescence intensity of EGFP or 4P-EGFP in the wild-type (WT), $\Delta 10DGC$, and ΔPDE strains. The 4P-EGFP production was recorded as a function of *c*-di-GMP (B) and EF-P (C) in the $\Delta 10DGC$ mutant strain. WspR and EF-P are expressed from an IPTG-inducible *tac* promoter. The fluorescence was measured when cells were grown to OD₆₀₀ = 1.0. The results are analyzed using two-way ANOVA for at least three independent experiments. ****P* < 0.001. a.u., arbitrary units. Error bars indicate the standard deviations (SD).

of that in the wild-type strain, respectively. Then, the $\Delta 10DGC$ mutant was transformed with an EGFP reporter plasmid harboring WspR, the expression of which was controlled by the isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible *tac* promoter. As shown in Fig. 5B, the translation of 4P-EGFP was positively correlated with the concentration of IPTG, while the translation of EGFP was not affected by *c*-di-GMP. In parallel, we also analyzed whether 4P-EGFP translation in $\Delta 10DGCs$ was impacted by *efp* under conditional expression. We observed that 4P-EGFP translation also occurred in an EF-P-dependent manner (Fig. 5C), suggesting that the translation of consecutive proline-containing proteins is modulated by EF-P, the activity of which was enhanced by *c*-di-GMP.

To further characterize the relationship between EF-P function and *c*-di-GMP sensing, we chose the two variant proteins EF-P^{K47A} and EF-P^{E69A} that almost lost their activity to bind with *c*-di-GMP. It was observed that the function of both EF-P^{K47A} and EF-P^{E69A} was reduced for modulating the translation of 4P-EGFP, biofilm formation, and motility compared with the wild-type EF-P in the presence of *c*-di-GMP (SI Appendix, Fig. S14). We continued to construct a $\Delta 10DGC\Delta efp/Pnat-efp^{E69A}$ mutant in which the EF-P^{E69A} was expressed from the native promoter in the pME2 plasmid. As expected, both EF-P and EF-P^{E69A} complemented the 4P-EGFP translation with similar activity in the absence of *c*-di-GMP (Fig. 6A). However, because EF-P^{E69A} was different from the wild-type EF-P, it could not sense *c*-di-GMP to

modulate the translation of 4P-EGFP (Fig. 6B). Our results indicated that the function of EF-P is enhanced in the presence of *c*-di-GMP, while EF-P still has a basal level of activity in the absence of *c*-di-GMP.

To further confirm the influence of *c*-di-GMP on EF-P, we first measured the transcriptional levels of *bfmS* and *csuD* via both qRT-PCR and β -galactosidase activity assays because both BfmS and CsuD contain consecutive prolines (SI Appendix, Fig. S15 A and B and Table S4). Although the transcriptional level of *csuD* was dependent on *c*-di-GMP, we observed that *bfmS* transcription was similar in the wild-type, $\Delta 10DGC$, and ΔPDE strains, suggesting that the transcription of *bfmS* was not dependent on *c*-di-GMP. Then, the BfmS protein levels in the wild-type, $\Delta 10DGC$, and ΔPDE strains were determined by fusion of the genes and their native promoters with EGFP. As shown in SI Appendix, Fig. S15C, the fluorescence signals of BfmS fusion proteins were dependent on both *c*-di-GMP and EF-P. However, when the consecutive prolines of BfmS (PPP³⁹⁷) were mutated to AAA, there was no difference in the fluorescence of BfmS^{AAA} fusion protein among the wild-type, $\Delta 10DGC$, and ΔPDE strains.

Discussion

As an important player in bacterial physiology, *c*-di-GMP not only determines the bacterial sessile-to-motile lifestyle transition but also binds to a range of effector components and controls

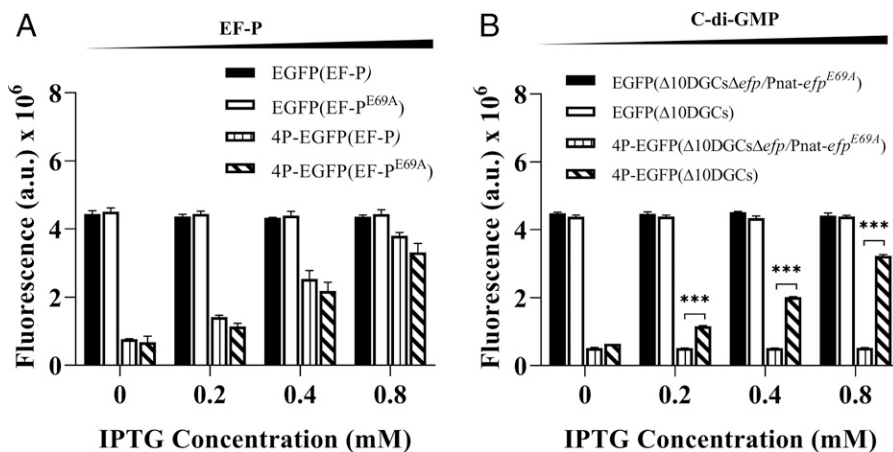


Fig. 6. Analysis of EF-P regulatory mechanism to modulate the translation of polyproline motif-containing proteins by sensing *c*-di-GMP. (A) The fluorescence intensity of EGFP and 4P-EGFP in the $\Delta 10DGCs\Delta efp/Pnat-efp^{E69A}$ mutant was recorded after transforming with a plasmid containing EF-P or EF-P^{E69A}, which was expressed from an IPTG-inducible *tac* promoter. (B) The fluorescence intensity of EGFP and 4P-EGFP in the $\Delta 10DGC$ mutant and $\Delta 10DGCs\Delta efp/Pnat-efp^{E69A}$ mutant was recorded after transforming with a plasmid containing WspR, which was expressed from an IPTG-inducible *tac* promoter. The results are analyzed using two-way ANOVA for at least three independent experiments. ****P* < 0.001. a.u., arbitrary units. Error bars indicate the standard deviations (SD).

diverse functions (41). *A. baumannii* ATCC 17978 harbors 12 potential *c*-di-GMP metabolic enzymes (*SI Appendix, Table S1*), and some of them were reported to be involved in the regulation of bacterial physiology, including biofilm formation and motility (24). However, the occurrence of GGDEF-EAL domain proteins does not imply the existence of *c*-di-GMP. For example, *Staphylococcus* has only remnants of *c*-di-GMP systems and cannot synthesize *c*-di-GMP, but the bacterium harbors a GGDEF domain protein to control biofilm formation through a *c*-di-GMP-independent mechanism (42). In this study, we isolated and characterized *c*-di-GMP in *A. baumannii* (*SI Appendix, Fig. S1A*) and found that this signal plays an important role in the regulation of biofilm formation, motility, and virulence (Fig. 1). Our findings provide additional evidence for the role of this kind of second messenger, which plays a vital role and is also widely present in bacteria.

Although there are 19 potential *c*-di-GMP metabolic enzymes in *Dickeya dadantii* strain EC1, it was found that only some of them (i.e., W909_14950, W909_10355, W909_02155, and W909_14945) were involved in *c*-di-GMP signaling (43), suggesting that not all GGDEF-EAL domain-containing proteins influence the intracellular *c*-di-GMP pool. In this study, we showed that only deletion of A1S_1695, A1S_2506, A1S_3692, and A1S_1254 caused apparent changes in the intracellular levels of *c*-di-GMP and phenotypes of *A. baumannii* under the investigated growth conditions (*SI Appendix, Fig. S6*). This might be a result of the compensatory mechanism and of functional redundancy, which were previously indicated to be responsible for the absence of phenotypic changes in some GGDEF-EAL protein deletion mutants of *Sinorhizobium meliloti* (1). Another explanation is that there could be a nonfunctional catalytic motif in these proteins that leads to loss of the *c*-di-GMP metabolic enzyme activity. For example, the GGDEF motif of A1S_2337 is replaced by NGDDF (*SI Appendix, Table S1*), resulting in the absence of DGC activity (24) (*SI Appendix, Fig. S5 F and G*). The structural model of RocR revealed that the helix loop 6 motif (DDFG (T/A)GYSS) of the EAL domain is essential for enzymatic activity (44), and the first glutamate residue in the EGVE motif is considered to be the catalytic base of the EAL domain (27). The helix loop 6 and the catalytic base of A1S_2422 were degenerated into RDFASSMYS and alanine, respectively (*SI Appendix, Table S1*), which might be the reason for the inactivation of the EAL domain of A1S_2422 (*SI Appendix, Fig. S2*). An additional possible explanation is that environmental factors also contribute to the deficiency in the activity of some *c*-di-GMP metabolic enzymes. For example, the PDE activity of PdeS is strongly inhibited by dephosphorylated EIIA^{Glc} in the presence of glucose (45). Together, these results suggest that the function of *c*-di-GMP metabolic enzymes is the outcome of evolution, metabolism, and environment combined.

Protein synthesis is dependent on ribosomal subunits, aminoacyl-transfer RNAs, messenger RNAs, and translation factors (46). EFs are a set of proteins that function at the ribosome to facilitate translational elongation from the formation of the first to the last peptide bond of a growing polypeptide during protein synthesis. There are 5 important EFs currently found in prokaryotes: EF-Tu, EF-Ts, EF-G, EF-P, and EF-4 (47–51). These factors play vital roles in the translation process and definitely modulate bacterial physiology. It was also revealed that some of these factors modulate the pathogenicity of pathogenic bacteria. Among them, EF-P is famous for its function in alleviating ribosome stalling during the translation of polyproline motifs (20, 50). In our study, we identified an EF-P (A1S_2419) that is essential for *A. baumannii* growth (Fig. 3A). Our research also

showed that A1S_2419 not only facilitates the translational elongation of proteins with consecutive prolines but also binds to *c*-di-GMP and influences the bacterial physiology and virulence of *A. baumannii* (Figs. 2 and 3).

Studies over the last 30 years show that *c*-di-GMP commonly controls various biological functions by interacting with diverse receptor or effector proteins (3–5, 9–18). Interestingly, different from these paradigms, the findings from this study have unveiled a new regulatory mechanism with which *c*-di-GMP could modulate bacterial physiology and virulence through multiple signaling pathways. On one hand, *c*-di-GMP regulates the target gene at the transcriptional level through a potential PilZ domain-containing effector or other unknown effectors (*SI Appendix, Figs. S8 and S15*). This conclusion was supported by the fact that *c*-di-GMP controls the transcriptional levels of various genes, such as *csuD* and *ompA* (*SI Appendix, Figs. S7 and S15 A and B and Table S2*). On the other hand, *c*-di-GMP regulates the target gene at the translational level through an EF-P (Fig. 5). Furthermore, *c*-di-GMP could control target genes such as *csuD* at both the transcriptional and translational levels (*SI Appendix, Fig. S15*). Intriguingly, some transcriptional regulators might also be the targets controlled by *c*-di-GMP and EF-P at the translational level because they contain three consecutive prolines (*SI Appendix, Table S4*). Together, these results suggest that *c*-di-GMP uses a complex and unique regulatory mechanism in *A. baumannii*.

A BLAST search with A1S_2419 revealed that this kind of EF-P is highly conserved in diverse bacterial species including members of the genera *Acinetobacter*, *Halomonas*, *Pseudoalteromonas*, *Vibrio*, *Xanthomonas*, and *Yersinia* (*SI Appendix, Table S5*). In conclusion, our data suggest that EF-P is a unique effector of *c*-di-GMP that is present in various bacterial pathogens. Our findings may inspire further studies to investigate roles and mechanisms of EF-P in *c*-di-GMP signaling systems in diverse bacterial genera.

Materials and Methods

Bacterial Growth Conditions and Virulence Assays. The bacterial strains used in this work are listed in *SI Appendix, Table S6*. *A. baumannii* ATCC 17978 strains were cultured at 37 °C in lysogeny broth (LB) Lennox. The following antibiotics were added when necessary: ampicillin, 100 µg/mL⁻¹; kanamycin, 50 µg/mL⁻¹; apramycin, 100 µg/mL⁻¹; and tetracycline, 12.5 µg/mL⁻¹. A cell line infection model was used for virulence assays following the methods indicated in *SI Appendix*.

Mutagenesis and Phenotype Analysis. *A. baumannii* ATCC 17978 was used as the parental strain to generate an in-frame deletion mutant of *efp*, following the methods indicated in *SI Appendix*. Motility and biofilm formation assays were performed using previously described methods, which are provided in *SI Appendix*.

Protein Purification and Analysis. Detailed descriptions are provided in *SI Appendix*. Briefly, open reading frames were amplified with the primers listed in *SI Appendix, Table S7* and cloned into the expression vector pET-28a. The fusion gene constructs were transformed into *E. coli* strain BL21. Affinity purification of fusion proteins and binding assays were performed using previously described methods, which are provided in *SI Appendix*.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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- S. Schäper *et al.*, Cyclic di-GMP regulates multiple cellular functions in the symbiotic alphaproteobacterium *Sinorhizobium meliloti*. *J. Bacteriol.* **198**, 521–535 (2015).
- S. Steiner, C. Lori, A. Boehm, U. Jenal, Allosteric activation of exopolysaccharide synthesis through cyclic di-GMP-stimulated protein-protein interaction. *EMBO J.* **32**, 354–368 (2013).
- D. Skotnicka *et al.*, CdbA is a DNA-binding protein and c-di-GMP receptor important for nucleoid organization and segregation in *Myxococcus xanthus*. *Nat. Commun.* **11**, 1791 (2020).
- B. J. Laventie *et al.*, A surface-induced asymmetric program promotes tissue colonization by *Pseudomonas aeruginosa*. *Cell Host Microbe* **25**, 140–152.e6 (2019).
- U. Römling, M. Y. Galperin, M. Gomelsky, Cyclic di-GMP: The first 25 years of a universal bacterial second messenger. *Microbiol. Mol. Biol. Rev.* **77**, 1–52 (2013).
- A. L. Lovering *et al.*, The structure of an unconventional HD-GYP protein from *Bdellovibrio* reveals the roles of conserved residues in this class of cyclic-di-GMP phosphodiesterases. *MBio.* **2**, e00163-11 (2011).
- M. W. Chen *et al.*, Structural insights into the regulatory mechanism of the response regulator RocR from *Pseudomonas aeruginosa* in cyclic Di-GMP signaling. *J. Bacteriol.* **194**, 4837–4846 (2012).
- X. Gao *et al.*, Functional characterization of core components of the *Bacillus subtilis* cyclic-di-GMP signaling pathway. *J. Bacteriol.* **195**, 4782–4792 (2013).
- F. Wang *et al.*, BrlR from *Pseudomonas aeruginosa* is a receptor for both cyclic di-GMP and pyocyanin. *Nat. Commun.* **9**, 2563 (2018).
- B. Y. Matsuyama *et al.*, Mechanistic insights into c-di-GMP-dependent control of the biofilm regulator FleQ from *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* **113**, E209–E218 (2016).
- C. R. Guzzo, R. K. Salinas, M. O. Andrade, C. S. Farah, PILZ protein structure and interactions with PILB and the FIMX EAL domain: Implications for control of type IV pilus biogenesis. *J. Mol. Biol.* **393**, 848–866 (2009).
- V. Shyp *et al.*, Reciprocal growth control by competitive binding of nucleotide second messengers to a metabolic switch in *Caulobacter crescentus*. *Nat. Microbiol.* **6**, 59–72 (2021).
- N. Tschowri *et al.*, Tetrameric c-di-GMP mediates effective transcription factor dimerization to control *Streptomyces* development. *Cell* **158**, 1136–1147 (2014).
- A. Kaczmarczyk *et al.*, Precise timing of transcription by c-di-GMP coordinates cell cycle and morphogenesis in *Caulobacter*. *Nat. Commun.* **11**, 816 (2020).
- Y. Deng *et al.*, Cis-2-dodecenoic acid receptor RpfR links quorum-sensing signal perception with regulation of virulence through cyclic dimeric guanosine monophosphate turnover. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 15479–15484 (2012).
- C. Yang *et al.*, *Burkholderia cenocepacia* integrates cis-2-dodecenoic acid and cyclic dimeric guanosine monophosphate signals to control virulence. *Proc. Natl. Acad. Sci. U.S.A.* **114**, 13006–13011 (2017).
- N. Sudarsan *et al.*, Riboswitches in eubacteria sense the second messenger cyclic di-GMP. *Science* **321**, 411–413 (2008).
- E. R. Lee, J. L. Baker, Z. Weinberg, N. Sudarsan, R. R. Breaker, An allosteric self-splicing ribozyme triggered by a bacterial second messenger. *Science* **329**, 845–848 (2010).
- A. L. Starosta *et al.*, Translational stalling at polyproline stretches is modulated by the sequence context upstream of the stall site. *Nucleic Acids Res.* **42**, 10711–10719 (2014).
- L. K. Doerfel *et al.*, EF-P is essential for rapid synthesis of proteins containing consecutive proline residues. *Science* **339**, 85–88 (2013).
- L. Peil *et al.*, Distinct XPPX sequence motifs induce ribosome stalling, which is rescued by the translation elongation factor EF-P. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 15265–15270 (2013).
- M. V. Rodnina, Translation in Prokaryotes. *Cold Spring Harb. Perspect. Biol.* **10**, a032664 (2018).
- K. R. Hummels, D. B. Kearns, Translation elongation factor P (EF-P). *FEMS Microbiol. Rev.* **44**, 208–218 (2020).
- I. Ahmad, E. Nygren, F. Khalid, S. L. Myint, B. E. Uhlin, A cyclic-di-GMP signalling network regulates biofilm formation and surface associated motility of *Acinetobacter baumannii* 17978. *Sci. Rep.* **10**, 1991 (2020).
- C. N. McQueary *et al.*, Extracellular stress and lipopolysaccharide modulate *Acinetobacter baumannii* surface-associated motility. *J. Microbiol.* **50**, 434–443 (2012).
- C. M. Harding, S. W. Hennon, M. F. Feldman, Uncovering the mechanisms of *Acinetobacter baumannii* virulence. *Nat. Rev. Microbiol.* **16**, 91–102 (2018).
- F. Rao, Y. Yang, Y. Qi, Z. X. Liang, Catalytic mechanism of cyclic di-GMP-specific phosphodiesterase: A study of the EAL domain-containing RocR from *Pseudomonas aeruginosa*. *J. Bacteriol.* **190**, 3622–3631 (2008).
- U. Römling, Rationalizing the evolution of EAL domain-based cyclic di-GMP-specific phosphodiesterases. *J. Bacteriol.* **191**, 4697–4700 (2009).
- A. J. Schmidt, D. A. Ryjenkov, M. Gomelsky, The ubiquitous protein domain EAL is a cyclic diguanylate-specific phosphodiesterase: Enzymatically active and inactive EAL domains. *J. Bacteriol.* **187**, 4774–4781 (2005).
- J. A. Gaddy, A. P. Tomaras, L. A. Actis, The *Acinetobacter baumannii* 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. *Infect. Immun.* **77**, 3150–3160 (2009).
- N. Pakharukova *et al.*, Structural basis for *Acinetobacter baumannii* biofilm formation. *Proc. Natl. Acad. Sci. U.S.A.* **115**, 5558–5563 (2018).
- A. Rajkovic, M. Ibba, Elongation factor P and the control of translation elongation. *Annu. Rev. Microbiol.* **71**, 117–131 (2017).
- K. R. Hummels *et al.*, Carbonyl reduction by YmfI in *Bacillus subtilis* prevents accumulation of an inhibitory EF-P modification state. *Mol. Microbiol.* **106**, 236–251 (2017).
- A. Rajkovic *et al.*, Cyclic rhamnosylated elongation factor P establishes antibiotic resistance in *Pseudomonas aeruginosa*. *MBio* **6**, e00823 (2015).
- R. Tollerson II, A. Witzky, M. Ibba, Elongation factor P is required to maintain proteome homeostasis at high growth rate. *Proc. Natl. Acad. Sci. U.S.A.* **115**, 11072–11077 (2018).
- J. Lassak *et al.*, Arginine-rhamnosylation as new strategy to activate translation elongation factor P. *Nat. Chem. Biol.* **11**, 266–270 (2015).
- S. Y. Kim *et al.*, The sensor kinase BfmS controls production of outer membrane vesicles in *Acinetobacter baumannii*. *BMC Microbiol.* **19**, 301 (2019).
- M. L. Liou *et al.*, The sensor kinase BfmS mediates virulence in *Acinetobacter baumannii*. *J. Microbiol. Immunol. Infect.* **47**, 275–281 (2014).
- E. Geisinger, R. R. Isberg, Antibiotic modulation of capsular exopolysaccharide and virulence in *Acinetobacter baumannii*. *PLoS Pathog.* **11**, e1004691 (2015).
- T. Yanagisawa *et al.*, *Neisseria meningitidis* translation elongation factor P and its active-site arginine residue are essential for cell viability. *PLoS One* **11**, e0147907 (2016).
- R. Hengge, Principles of c-di-GMP signalling in bacteria. *Nat. Rev. Microbiol.* **7**, 263–273 (2009).
- L. M. Holland *et al.*, A staphylococcal GGDEF domain protein regulates biofilm formation independently of cyclic dimeric GMP. *J. Bacteriol.* **190**, 5178–5189 (2008).
- Y. Chen *et al.*, Systematic analysis of c-di-GMP signaling mechanisms and biological functions in *Dickeya zeae* EC1. *MBio* **11**, e02993 (2020).
- T. R. Barends *et al.*, Structure and mechanism of a bacterial light-regulated cyclic nucleotide phosphodiesterase. *Nature* **459**, 1015–1018 (2009).
- K. Heo *et al.*, Sugar-mediated regulation of a c-di-GMP phosphodiesterase in *Vibrio cholerae*. *Nat. Commun.* **10**, 5358 (2019).
- T. E. Dever, E. Gutierrez, B. S. Shin, The hypusine-containing translation factor eIF5A. *Crit. Rev. Biochem. Mol. Biol.* **49**, 413–425 (2014).
- D. Kavaliuskas, P. Nissen, C. R. Knudsen, The busiest of all ribosomal assistants: Elongation factor Tu. *Biochemistry* **51**, 2642–2651 (2012).
- B. J. Burnett *et al.*, Elongation factor Ts directly facilitates the formation and disassembly of the *Escherichia coli* elongation factor Tu-GTP-aminocyl-tRNA ternary complex. *J. Biol. Chem.* **288**, 13917–13928 (2013).
- H. Yamamoto *et al.*, EF-G and EF4: Translocation and back-translocation on the bacterial ribosome. *Nat. Rev. Microbiol.* **12**, 89–100 (2014).
- S. Ude *et al.*, Translation elongation factor EF-P alleviates ribosome stalling at polyproline stretches. *Science* **339**, 82–85 (2013).
- Y. Qin *et al.*, The highly conserved LepA is a ribosomal elongation factor that back-translocates the ribosome. *Cell* **127**, 721–733 (2006).