Title: The cNMP Domain of Xanthomonas campestris Global Regulator

Clp Defines a New Class of C-di-GMP Effectors

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Abstract

The widely conserved second messenger cyclic diguanosine monophosphate (c-di-GMP) plays a key role in quorum sensing (QS) dependent production of virulence factors in *Xanthomonas campestris pv. campestris* (*Xcc*). The detection of QS diffusible signal factor (DSF) by the sensor RpfC leads to the activation of response regulator RpfG, which activates virulence gene expression by degrading c-di-GMP. Here, we show that a global regulator in the *Xcc* QS regulatory pathway, Clp, is a c-di-GMP effector. C-di-GMP specifically binds to Clp with high affinity and induces allosteric conformational changes that abolish the interaction between Clp and its target gene promoter. Clp is similar to the cAMP binding proteins Crp and Vfr, and contains a conserved cNMP binding domain. Using site-directed mutagenesis, we found that the cNMP binding domain of Clp contains a glutamic acid residue (E99) that is essential for c-di-GMP binding. Substituting the residue with serine (E99S) resulted in decreased sensitivity to changes in the intracellular c-di-GMP level and attenuated bacterial virulence. These data establish the direct role of Clp in the response to fluctuating c-di-GMP levels and depict a novel mechanism by which QS links the second messenger with the *Xcc* virulence regulon.
INTRODUCTION

The nucleotide signaling molecule cyclic diguanosine monophosphate (c-di-GMP) has recently emerged as a widely conserved second messenger implicated in the regulation of various biological functions in bacteria, including cellulose biosynthesis (24), bacterial motility (27), biofilm formation (12), and extracellular virulence factor production (25). The intracellular level of c-di-GMP is influenced by the opposite activities of the diguanylate cyclases via GGDEF domain proteins that synthesize c-di-GMP and the phosphodiesterases via EAL or HD-GYP domain proteins that degrade this signaling molecule (15). In accordance with the central role of c-di-GMP in bacterial physiology, the three domains associated with c-di-GMP metabolism (GGDEF, EAL, and HD-GYP) are broadly distributed in a wide range of bacteria species (3, 15, 23). Notably, these domains are commonly linked to various signal sensing or detection domains, suggesting that various environmental cues modulate bacterial physiology by influencing the rate of c-di-GMP synthesis and hydrolysis. The characterization of proteins that sense and detect changes in the c-di-GMP level is key to understanding how this ubiquitous second messenger modulates such diverse biological functions. Several types of c-di-GMP receptors have been unveiled in recent years, including several enzymes and proteins containing a PilZ domain (1), PleD and PelD containing the GGDEF domain with an RXXD motif (2, 19), and LapD containing a degenerated EAL domain (22). These effectors are not directly involved in transcriptional control because they do not contain DNA binding domains. Recently, the transcription factor FleQ of
Pseudomonas aeruginosa, which interacts with c-di-GMP, was reported. FleQ suppresses EPS biosynthesis by binding to the pel promoter, and this binding is inhibited by c-di-GMP (11).

C-di-GMP is implicated in the regulation of virulence in Xanthomonas campestris pv. campestris (Xcc). The pathogen produces a range of extracellular virulence factors, including protease, cellulose, pectinase, and extracellular polysaccharide (EPS) (3, 10). The production of these virulence factors is regulated by the diffusible signal factor (DSF)-mediated quorum sensing (QS) mechanism and the RavS/RpsR two component system. In the QS system, detection of the DSF involves the sensor RpfC, which transduces the QS signal to its cognate response regulator, RpfG, through a conserved phosphorelay mechanism (8). Activated RpfG functions as a phosphodiesterase, degrading c-di-GMP to GMP (25). In the RavS/RpsR system, the sensor RavS contains a PAS domain implicated in hypoxia sensing, whereas the response regulator RavR acts as a c-di-GMP degrading phosphodiesterase (6). In both systems, c-di-GMP degradation results in enhanced transcriptional expression of the global regulator Clp, which positively regulates virulence factor production (6, 7). However, how Clp detects and responds to the RpfG- and RavR-mediated changes of the steady-state level of c-di-GMP is unclear.

Clp is a global regulator that shares a conserved cNMP binding domain with the well-characterized cAMP nucleotide receptors Crp of Escherichia coli and Vfr of Pseudomonas aeruginosa (7). Peptide sequence alignment has revealed that Clp differs from Crp and Vfr in
only one or two of the six conserved residues implicated in cAMP binding. In this study, we demonstrate that Clp interacts with c-di-GMP with high affinity and that this interaction abolishes the ability of Clp to bind to its target gene promoter. We also demonstrate that the single amino acid variation (E99) in the cNMP binding domain of Clp, which distinguishes Clp from Crp (corresponding residue S83) and Vfr (corresponding residue S88), is the key residue defining the ability of Clp to interact with c-di-GMP. The cNMP-binding domain protein from *Stenotrophomonas maltophilia*, which is homologous to Crp and Vfr but shares the conserved E99 residue with Clp, also binds c-di-GMP, but not cAMP. The evidence suggests that a subfamily of cNMP-binding domain proteins may be c-di-GMP effectors with the conserved E99 residue as a signature amino acid residue.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *Xcc* strains were grown at 30°C in YEB medium (29) unless otherwise stated. *E. coli* strains were grown at 37°C in LB medium. Antibiotics were added at the following concentrations when required: 100 µg/ml kanamycin, 50 µg/ml rifampicin, 10 µg/ml tetracycline. X-gluc (5-bromo-4-chloro-3-indolyl-β-glucopyranoside) was included in the medium (60 µg/ml) to detect GUS (β-glucuronidase) activity. The synthesis of DSF was described previously (29). The signaling molecule was added to the medium at a final concentration of 5 µM when necessary.
Generation of in-frame deletion mutants and in trans expression constructs. A spontaneous rifampicin-resistant derivative of strain 8004 was used as the parental strain for generating deletion mutants. In-frame deletion mutants of rpfG and clp were generated using the primers listed in Supplementary Table S1 and following the previously described methods (9). For in trans expression, the coding regions of the corresponding proteins were amplified by PCR using the primers listed in Table S1 and cloned separately under the control of the lac promoter in the expression vector pDSK519. The resulting constructs were transferred into Xcc strains through triparental mating.

Quantitative determination of GUS activity and extracellular polysaccharide (EPS) production. Bacterial cells were collected by centrifugation at 14,000 rpm for 10 min, and the total soluble protein was prepared by sonication. GUS enzyme activity was determined quantitatively according to the standard protocol (14). The substrate 4-methylumbelliferyl-β-D-gucuronide was used for fluorometric assays on a HITACHI F1000 fluorescence spectrophotometer (Sigma) with excitation at 365 nm and emission at 455 nm. The data is reported as the averages from at least three independent repeats and defined as picomoles of methyl umbelliferone produced per minute per microgram of total soluble protein.

To analyze EPS production, supernatants were collected from overnight bacterial cultures (10 ml, OD$_{600}$=2.0) after centrifugation as described above. Two volumes of absolute ethanol were added to the supernatants, and the mixtures were kept at -20°C for half an hour. The
precipitated EPS molecules were spun down and dried at 55°C overnight before determining the dry weight.

**RNA extraction and RT-PCR analysis.** Total RNA was isolated from fresh bacterial cultures. Briefly, bacterial cells were harvested at OD$_{600}$ = 2.0 by centrifugation at 4°C for 4 min at 10,000 rpm. RNA was purified using RNeasy mini columns (Qiagen) following the manufacturer’s protocol. After digestion with DNase I (Promega) to remove contaminating genomic DNA residues, the RNA samples were re-purified using an RNeasy column to remove excessive enzyme. The quantity and purity of the RNA was determined by agarose gel electrophoresis and spectrometry. RT-PCR analysis was performed using the OneStep RT-PCR Kit (Qiagen) following the manufacturer’s instructions. The primers used for RT-PCR analysis are listed in Table S1. The density of the PCR band was determined using Image J (http://rsb.info.nih.gov/ij/).

**Site-directed mutagenesis of clp and in situ substitution.** The site-directed mutagenesis of clp was performed using the Quickchange® site-directed mutagenesis kit following the manufacturer’s protocol (Stratagene). The coding region was amplified using the primers Clp-F2 and Clp-R2 (Table S1) and cloned into the vector pGEMT-easy to generate pGEM-clp, which was used for subsequent site-directed mutagenesis. Based on the sequence alignment with Crp and Vfr, residues E99 and T149 of Clp were changed to serine using the primer pairs E99S-FOR/E99S-REV and T149S-FOR/T149S-REV (Table S1), respectively. After verification by DNA sequencing, the mutated clp alleles were ligated with pDSK519 to...
generate expression constructs pDSK-clpE99S and pDSK-clpT149S, respectively, for in trans expression in Xcc.

For in situ substitution of wild-type clp in Xcc, the clp alleles were subcloned into the SmaI site of suicide vector pK18mobsacB. Resulting constructs pK-clpE99S and pK-clpT149S were separately mobilized into Xcc wild-type strain 8004 by triparental mating. The transformants were selected on LB medium supplemented with rifampicin and kanamycin. A second selection to remove the vector was performed on YEB medium containing 5% (w/v) sucrose and rifampicin. The corresponding substitutions in the Xcc genome were confirmed by PCR amplification of the Clp coding region and DNA sequencing.

In trans expression of c-di-GMP metabolic enzymes. To modulate the c-di-GMP level in Xcc strains, PA5487 and PA3947, which encode a diguanylate cyclase and a phosphodiesterase (16), respectively, were amplified from Pseudomonas aeruginosa strain PAO1 using the primer pairs PA5487-FOR/PA5487-REV and PA3947-FOR/PA3947-REV (Table S1), respectively. After digestion with XbaI and EcoRI, the PCR products were cloned under the control of the lac promoter in the plasmid vector pDSK519 to generate expression constructs pDSK-PA5487 and pDSK-PA3947, respectively. The constructs were verified by DNA sequencing and mobilized separately into Xcc strains by triparental mating. The resulting transformants were selected on LB medium supplemented with rifampicin and kanamycin.
Protein purification. To purify Clp, variants ClpE99S and ClpT149S, and SmClp, their coding regions were amplified using the corresponding primer pairs listed in Table S1 and cloned into the expression vector pGEX-6p-1 (Novagen). The resulting constructs (pGEX-clp, pGEX-clpE99S, pGEX-clpT149S, and pGEX-Smclp; Table S1) were transformed into host strain *E. coli* BL21. After sequencing confirmation, these four proteins were expressed and purified separately using Glutathione Sepharose™ 4B columns according to the procedures recommended by the manufacturer (Amersham). Protein purity was checked by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining. Purified proteins were dialyzed against 20 mM Tris buffer (pH 7.3) containing 50 mM NaCl using 3000 molecular weight cut-off centrifugal filter devices (Millipore, Bedford, MA). The samples were stored as aliquots in 50% (v/v) glycerol at -20°C until use.

Electrophoretic mobility shift assay (EMSA). DNA probes for EMSA were obtained by PCR amplification using the primer pairs listed in Table S1. The PCR fragments were purified using the QIAquick PCR purification kit (Qiagen) and labeled using a DIG-end-labeling kit when necessary. The DNA-protein binding reaction was performed according to the manufacturer’s instructions (Roche). A 6% polyacrylamide gel was used for separation of the DNA-protein complex. After UV cross-linking, the DIG-labeled probes were detected in the membrane using a DIG Luminescent Detection Kit (Roche).

Circular dichroism (CD) spectroscopy and isothermal titration calorimetry (ITC). Far-UV CD analysis of Clp was carried out on a JASCO J-810 spectropolarimeter as previously
described (29). Clp and c-di-GMP were added at a final concentration of 20 µM. The ITC measurements were obtained using a VP-IPC calorimeter following the manufacturer’s protocol (MicroCal, Northampton, MA). In brief, 5 µl aliquots of nucleotide solution (500 µM) were injected at 5-min intervals via a 300 µl syringe into the sample cell containing 1.4 ml of Clp or its homologue (20 µM) with constant stirring at 290 rpm, and the heat changes accompanying these additions were recorded. The protein samples were extensively dialyzed against Tris-HCl buffer (20 mM Tris-HCl pH 7.3, 50 mM NaCl) before titration. The nucleotide solution was prepared directly in Tris-HCl buffer and the titrate solutions were degassed under a vacuum prior to loading for ITC. The titration experiment was repeated at least twice, and the data were calibrated with a buffer control and fitted with the one-site model to determine the binding constant (Ka) using the MicroCal ORIGIN V7.0 software.

**Virulence assay.** The virulence assay was conducted by the inoculation of Xcc strains onto Chinese cabbage following the vacuum infiltration method with minor modifications (4). Briefly, cabbage stem tissue was cut into pieces approximately 2 x 2 cm in size, which were then immersed in an overnight culture of bacterial suspension (OD\textsubscript{600} = 2.0) prior to 2-min vacuum exposure to facilitate bacterial infiltration. The treated stem tissues were then transferred to an incubator at 30°C with humidity at around 90%. Symptom development was monitored daily, and the photographs were taken 4 days later. The experiment was repeated at least three times in duplicate.
RESULTS

C-di-GMP is a specific Clp ligand. The available evidence suggests that Clp is a global transcriptional regulator located downstream of the RpfC/RpfG two-component system in the DSF pathway (7). Based on this model, the intracellular level of DSF is under a threshold level at low cell density, which favors c-di-GMP accumulation as its degradation enzyme RpfG exists in its inactive form. On the other hand, at high cell density, the accumulated DSF activates RpfG through the sensor RpfC, resulting in c-di-GMP degradation and the accumulation of GMP. To understand the mechanism by which Clp and c-di-GMP act in the DSF signaling pathway, we tested whether Clp can detect c-di-GMP or its related nucleotides as ligands using isothermal titration calorimetry (ITC). Clp was expressed as a glutathione S-transferase (GST) fusion protein, which was used in subsequent analysis because the solubility of recombinant Clp separated from GST is poor. In the ITC analysis, the Clp fusion protein solution was titrated with c-di-GMP and its derivatives at 20°C and the heat released upon binding measured. The results showed a strong interaction between Clp fusion protein and c-di-GMP (Fig. 1A). As expected, no physical interaction was observed between the control protein, GST, and c-di-GMP (data not shown). The binding isotherm data suggested that Clp tightly binds c-di-GMP in 1:1 stoichiometry with an estimated dissociation constant ($K_d$) of $1.61 \pm 0.22$ M. In contrast, the addition of cAMP, even at a level 20-fold higher than Clp fusion protein, did not cause a similar heat release as c-di-GMP (Fig. 1B). Similarly, no
molecular interaction was observed between Clp and GMP or GTP (Supplementary Fig. S1A and S1B), indicating that they are not the cognate signal ligands of Clp.

C-di-GMP inhibits the formation of the protein-promoter complex consisting of Clp and its target promoter PengXCA. Clp is a global regulator with a DNA binding domain at its c-terminal (7). To further analyze the impact of c-di-GMP on Clp functionality, we amplified the promoter region of engXCA and performed an electrophoretic mobility shift assay (EMSA). EngXCA encodes an extracellular endoglucanase (5) that contributes to Xcc virulence through its cellulolytic activity. Previous analyses showed that engXCA belongs to the DSF- and Clp-regulon (7, 9) and is under the direct control of Clp (13). When purified Clp fusion protein was incubated with a DIG-labeled engXCA promoter (PengXCA) fragment (382 bp), protein-DNA complexes formed in a Clp-dosage-dependent manner (Fig. S2A). In contrast, no band shift was observed in the negative control lacking protein or with GST (Fig. S2A). The specificity of Clp fusion protein binding to PengXCA was further verified using competitor DNA. The intensity of the shifted band was diminished in the presence of unlabeled PengXCA (Fig. S2B) but not in the presence of the unlabeled prt1 promoter (Fig. S2B). A previous study showed that prt1, which encodes a protease, is not directly regulated by Clp (13). Under the same experimental conditions, we tested whether the ligand c-di-GMP influences the formation of the protein-promoter complex consisting of Clp and PengXCA. The addition of c-di-GMP, at either 10 µM or 100 µM, abolished the ability of Clp to form a complex with its target promoter (Fig. 2A). Consistent with the results of the ITC analysis, the
addition of GMP and GTP, up to 100 µM, had no effect on the Clp-promoter interaction (Fig. 1A).

**C-di-GMP acts as an allosteric regulator of Clp.** To understand how c-di-GMP might inhibit the formation of the Clp-promoter complex, we used circular dichroism (CD) spectroscopy to determine the effect of c-di-GMP on the conformational structure of Clp. The CD spectrum of the Clp protein showed intensive negative ellipticity from 200 nm to 230 nm (Fig. 2B), suggesting a large unordered contribution and a small, but detectable, contribution of the α-helical structure (33). In contrast, the addition of c-di-GMP resulted in notable conformational changes in Clp, including decreased intensity of the negative ellipticity from 200 nm to 230 nm and a shifted peak of positive ellipticity (Fig. 2B), indicating reduced content of the α-helical structure and increased β-sheet content, respectively (33). As a negative control, the addition of c-di-GMP did not alter the CD spectra of the GST protein (Fig. 2C). Taken together, the results demonstrate that c-di-GMP binding induces allosteric conformational changes in Clp, which might consequently affect the affinity of the transcription factor to its target promoter. In addition, the findings suggest that c-di-GMP shares a mechanism of action with cAMP, which is known as the allosteric regulator of Crp (32).

**Modulation of intracellular c-di-GMP levels alters the expression pattern of engXCA.** The above data suggest that c-di-GMP acts as an inhibitor of Clp-dependent virulence gene expression. To test this possibility, we fused the GUS coding region to the engXCA promoter,
generating reporter construct pL3PengXCA-GUS. First, we tested the expression pattern of
engXCA in the presence and absence of functional Clp gene. The reporter construct
pL3PengXCA-GUS was introduced into the clp in-frame deletion mutant Δclp and its wild-
type strain 8004. The mutation of clp did not affect Xcc growth (data not shown), but
diminished the expression of engXCA (Fig. 3A). In agreement with the previous report (13),
the data indicate that Clp is a key transcriptional factor in the regulation of engXCA
expression under the experimental conditions used in this study.

Next, we determined whether a fluctuation in the intracellular level of c-di-GMP affects the
transcriptional expression of the engXCA operon in Xcc. For this purpose, we cloned the
genes PA5487 and PA3947 from Pseudomonas aeruginosa for in trans expression in Xcc.
Previously, PA5487 was shown to encode a diguanylate cyclase that synthesizes c-di-GMP,
and PA3947 encodes a phosphodiesterase that degrades the second messenger (16). In the
wild-type Xcc carrying the vector pDSK519 alone, roughly 2500 units of β-glucuronidase was
detected. In contrast, in trans expression of the c-di-GMP synthase encoded by PA5487
resulted in an approximate 10-fold reduction in engXCA transcription in Xcc (Fig. 3B), which
is consistent with the notion that high cellular c-di-GMP levels suppress virulence gene
expression. To test the impact of decreased c-di-GMP levels, we used the RpfG null mutant
ΔrpfG, which should have a higher intracellular c-di-GMP level than wild-type Xcc (25).
Consistent with this expectation, increased engXCA expression was observed in ΔrpfG
expressing the c-di-GMP degradation enzyme encoded by PA3947 compared to the control
expression of PA3947 in strain ΔrpfG resulted in increased engXCA transcript (Fig. 3C).

The conserved amino acid residues implicated in the cNMP binding domain are required for the full activity of Clp. Peptide sequence alignment revealed that Clp shares approximately 45% and 48% of its amino acids with Crp and Vfr, respectively. Notably, of the six amino acid residues implicated in Crp binding to cAMP (18, 31), four are conserved in Clp (G87, E88, R98, T148; the varied residues: E99, T149) (7). Compared to Vfr, Clp differs in only one of these key residues (E99) (7). To test the impact of this variation in the cAMP binding motif on the transcription factor activity of Clp, we generated two clp alleles (clpE99S, clpT149S) by site-directed mutagenesis. Wild-type clp and its alleles were cloned under the control of the lac promoter in the plasmid vector pDSK519 for in trans expression. In trans expression of wild-type clp in the deletion mutant Δclp resulted in increased engXCA expression compared to the wild-type control, whereas expression of the clp variants in the same mutant, Δclp, only partially restored engXCA expression (Fig. 4A). In addition, a similar pattern was observed when we tested the impact of these Clp variants on EPS production (Fig. 4B), which is another trait of Xcc regulated by the DSF- and Clp-regulon (7, 9). The data suggest that the key residues in the cNMP binding domain are associated with the full activity of Clp. Given that the transcription factor activity of Clp does not require the presence of c-di-GMP (13) and that the residues involved in cAMP binding are located at the interface of two subunits of the Crp dimer (20, 21), we speculate that E99S and T149S substitution may
influence Clp dimer association or stability, which consequently affects its activity as a transcription factor.

Substitution of a key amino acid residue (E99) in the cNMP domain of Clp diminishes its response to c-di-GMP. To determine the effect of a key amino acid variation in the cNMP domain on ligand binding, we purified the E99S and T149S variant proteins using the same method as Clp purification. In the absence of the c-di-GMP ligand, E99S and T149S bound to the engXCA promoter similar to wild-type Clp; the T149S substitution did not seem to influence its response to the c-di-GMP ligand as the addition of 5 μM or 50 μM c-di-GMP diminished the promoter binding activity of both Clp and T149S (Fig. 5A). However, the E99S variant still formed a protein-DNA complex in the presence of c-di-GMP (Fig. 5A). The size of the E99S-DNA complex formed in the presence of c-di-GMP was smaller than that formed in the absence of second messenger (Fig. 5A). One plausible explanation is that addition of c-di-GMP to the E99S variant substantially reduced its affinity for one of the two Clp binding sites presented in the engXCA promoter (Fig. S3) (13).

To verify the EMSA findings, we tested the effect of over-expression of the c-di-GMP synthase encoded by PA5487 of P. aeruginosa. We transferred the altered clp alleles (clpE99S and clpT149S) to the chromosome of strain 8004 to replace wild-type clp by in situ substitution and used the resulting Xcc derivatives for further analysis. Increased c-di-GMP content due to the expression of PA5487 decreased engXCA expression in strains with wild-type clp or its allele clpT149S by roughly 10-fold, whereas expression of PA5487 in the strain
containing the clpE99S allele only reduced engXCA expression by less than 20% (Fig. 5B).

Consistently, the virulence assay showed that production of c-di-GMP due to the expression of PA5487 in the strain with wild-type clp or its altered allele clpT149S resulted in a loss of virulence in cabbage, whereas the expression of PA5487 in the strain with the clpE99S allele failed to stop bacterial infection; the maceration symptom was less extensive without PA5487 compared to its parental strain E99S (Fig. 5C). Taken together, these data suggest that E99 is a key amino acid residue that specifies the ability of Clp to respond to c-di-GMP, whereas the substitution of T149 with serine affects only the transcription factor activity of Clp and has no influence on its response to the inhibitory ligand c-di-GMP.

To further confirm the interaction between ClpE99S and c-di-GMP, ITC was carried out. The ClpE99S fusion protein solution was titrated with c-di-GMP at 20°C, and the heat released upon binding was measured. However, no physical interaction was observed between ClpE99S and c-di-GMP (Fig. S4).

The Clp homologue from Stenotrophomonas maltophilia also interacts with c-di-GMP.

Interestingly, the alignment of the amino acid sequences of Clp homologues revealed that these regulatory proteins can be grouped into two categories based on the amino acid aligned with E99 of Clp: one group shares the conserved residue serine (S), represented by Crp and Vfr, and the other shares the conserved residue glutamic acid (E), including a range of Clp homologues from various Xanthomonas species and Stenotrophomonas maltophilia (Fig. 6A).
GMP, we purified the Clp homologue (SmClp) from *S. maltophilia* for ITC. SmClp did not interact with cAMP but bound strongly to c-di-GMP with a *K*<sub>d</sub> of 2.16 ± 0.54·10<sup>-7</sup>M (Fig. 6B and 6C).

**DISCUSSION**

One of the intriguing puzzles in the *Xcc* DSF-dependent QS system is the mechanism by which QS signal sensing is coupled to intracellular regulatory networks through the second messenger c-di-GMP and global regulator Clp (10). In this study, we present several lines of evidence that Clp is a novel regulator of the c-di-GMP response. First, Clp specifically binds c-di-GMP with a high affinity (*K*<sub>d</sub> = 1.61 ± 0.22·10<sup>-7</sup>M). Second, c-di-GMP inhibits the binding of Clp to the *engXCA* promoter. Third, c-di-GMP induces intensive allosteric conformational changes in Clp. Fourth, increasing the intercellular content of c-di-GMP by over-expressing c-di-GMP synthase down-regulates virulence gene expression and attenuates bacterial virulence. These data, together with previous findings on the DSF-dependent QS, including the in transcriptional self-regulation of Clp (7), depict a detailed DSF signaling model in the regulation of bacterial virulence (Fig. 7). In this model, a basal level of DSF is produced at a low cell density because RpfF is bound to RpfC (8); RpfG is in its inactive state and c-di-GMP is maintained at a relatively high level that inactivates Clp by direct binding (Fig. 7A). When bacterial cells reach a high population density, the detection of DSF by RpfC results in the release of RpfF and phosphorylation of RpfG, which leads to increased DSF production.
and the degradation of c-di-GMP (7, 25). Consequently, the cellular level of free Clp increases, and the regulator acts as a positive transcription factor to induce its own gene transcription and virulence gene expression (Fig. 7B).

While this manuscript was being prepared, a paper was published online that shows the inhibitory effect of c-di-GMP on the binding of the Clp homologue from Xanthomonas axonopodis pv. citri to the synthetic DNA fragment with the conserved Crp binding site (17).

In addition to confirming that the Clp of Xcc is also an effector protein of c-di-GMP, this study has demonstrated the role of a key residue associated with ligand binding in the DSF-dependent modulation of virulence factor production under both in vitro and in vivo conditions. The identification of Clp as a c-di-GMP-responsive regulator has added a new member to the expanding superfamily of c-di-GMP effectors. Several types of c-di-GMP effectors have been identified in recent years, including PilZ, PleD, PelD, LapD, and FleQ (1, 11, 19, 26). Clp shares little homology with these known effector proteins at the amino acid level and differs from others in domain structure (data not shown). In contrast to the previously known c-di-GMP effectors, which require c-di-GMP for their functionality, Clp expands the regulatory mechanism of c-di-GMP by acting as a positive global regulator with c-di-GMP as an inhibitory ligand in the modulation of a large regulon encoding various biological functions, which includes over 300 genes (7). The identification of Clp as a c-di-GMP responsive global regulator provides new insight into how Xcc can couple the signal
inputs from various c-di-GMP metabolic mechanisms, such as RpfC/RpfG and RavS/RavR (6, 9, 28), to its virulence regulon.

Crystal structure analysis of the Crp-cAMP complex revealed that serine 83 (S83, the corresponding residue of E99 in Clp) within the conserved cNMP binding domain of Crp may play a key role in protein-ligand interactions by forming a hydrogen bond with the phosphate oxygen of cAMP through its functional hydroxyl group (20). Substitution of the S83 in Crp with a similar amino acid, threonine (S83T), to retain a hydroxyl group for hydrogen bonding, does not significantly affect the ability of Crp to respond to cAMP, whereas replacement of S83 with alanine, which has a methyl functional group and is unable to form a hydrogen bond with the ligand, abolishes Crp activity (21). Intriguingly, wild-type Clp contains a glutamic acid (E99) at the position corresponding to residue S83 of Crp. Glutamic acid is different from serine in that it contains a carbonyl functional group instead of a hydroxyl group and, thus, is unable to donate a hydrogen atom to hydrogen bond formation with phosphate oxygen. In this study, we found that the replacement of E99 with serine (E99S) in Clp deprives it of its ability to bind to c-di-GMP in vitro and results in a loss of sensitivity to c-di-GMP inhibition under in vivo conditions. These results establish the role of the conserved cNMP binding domain, which contains the residue E99, in the Clp and c-di-GMP interaction. The results also suggest that the role of residue E99 in ligand binding should be different from its counterpart Crp in cAMP binding due to the different physicochemical properties of their functional groups.
Furthermore, we provide evidence that the Clp-type c-di-GMP receptors are likely widely conserved in other bacterial species. The intriguing findings that Crp is the receptor for cAMP and Clp is the receptor for c-di-GMP encouraged us to conduct a sequence alignment of the proteins with cNMP binding domains (Fig. 6A) and biochemical analysis of the Clp homologue (SmClp) from *S. maltophilia* (Fig. 6B). The results indicate that the amino acid residue at the position corresponding to E99 in Clp may play a key role in determining ligand specificity. The cAMP receptors, represented by Crp and Vfr, share the conserved residue serine (S), whereas the c-di-GMP receptors Clp and SmClp share the conserved residue glutamic acid (E) (Fig. 6A). These findings suggest that the conserved residue E99 in Clp represents a signature residue of the c-di-GMP effectors with cNMP binding domains. The notion is further strengthened by the finding that the Clp homologue of *Xanthomonas axonopodis pv. citri*, which was recently shown to be a c-di-GMP effector (17), also contains the conserved E99 residue (Fig. 6A).

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<td>Wild-type strain 8004, Rif&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>He et al., 2006b</td>
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<td>Mutant ∆clp harboring construct pDSK-clpE99S, Rif&lt;sup&gt;r&lt;/sup&gt;, Kan&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>Strain 8004 derivative with clp replaced in situ by clpT149S</td>
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<td>Mutant E99S harboring vector pDSK-PA5487, Rif(^r), Kan(^r)</td>
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**E. coli**

- **DH5α**: supE44 ΔlacU169(F80lacZDMM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 Laboratory collection
- **BL21**: FompT hsdSB(rB\(^{−}\)mB\(^{−}\)) gal dcm \(\lambda\)(DE3) Laboratory collection
- **RK2013**: Triparental mating helper strain, Kan\(^r\) Laboratory collection

**Plasmid**

- **pDSK519**: Broad host range cloning vector, Kan\(^r\) Laboratory collection
- **pGEX-6P-1**: GST-fusion protein expression vector, Amp\(^r\) H. W. Song
- **pGEM-4-GUS**: Plasmid pGEM-4 containing promoterless gusA, Amp\(^r\) Present study
- **pLAFR3**: Broad host range cloning vector, Tec\(^r\) Laboratory collection
pGEMT-easy  Plasmid vector for cloning PCR fragment, Amp<sup>r</sup>  Promega
pK18mobsacB  Sucrose-sensitive suicide plasmid, Kan<sup>r</sup>  Schafer et al., 1994
pL3PengXCA-GUS  DSF sensor with gusA under the control of the engXCA promoter  Wang et al., 2004
pDSK-clp  clp placed under the control of the lac promoter in vector pDSK519, Kan<sup>r</sup>  Present study
pDSK-clpE99S  clpE99S placed under the control of the lac promoter in vector pDSK519, Kan<sup>r</sup>  Present study
pDSK-clpT149S  clpT149S placed under the control of the lac promoter in vector pDSK519, Kan<sup>r</sup>  Present study
pDSK-PA3947  PA3947 placed under the control of the lac promoter in vector pDSK519, Kan<sup>r</sup>  Present study
pDSK-PA5487  PA5487 placed under the control of the lac promoter in vector pDSK519, Kan<sup>r</sup>  Present study
pGEM-clp  clp cloned in pGEMT-easy, Amp<sup>r</sup>  Present study
pGEX-clp  clp cloned in vector pGEX-6P-1 for protein purification, Amp<sup>r</sup>  Present study
pGEX-Smclp  The clp homologue from *Stenotrophomonas maltophilia* cloned in vector pGEX-6P-1 for protein purification, Amp<sup>r</sup>  Present study
pGEX-clpE99S  clpE99S cloned in vector pGEX-6P-1 for protein purification, Amp<sup>r</sup>  Present study
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Figure Legends

FIG. 1. Isothermal titration calorimetry (ITC) analysis of (A) c-di-GMP and (B) cAMP binding to Clp. The top portion of each panel shows the ITC titration of 20 µM Clp with 5 µl aliquots of 500 µM c-di-GMP or 10 mM cAMP in Tris-HCl buffer at 20°C. The lower portion of each panel shows the binding isotherm for the titration as described in the upper portion, where the solid line is the best fit of the data to a one-site binding model.

FIG. 2. The effect of c-di-GMP on the conformational structure and promoter binding activity of Clp. (A) EMSA analysis of the impact of c-di-GMP on Clp binding to the engXCA promoter. Dig-labeled promoter fragments were incubated with purified Clp proteins in the presence or absence of nucleotide as indicated. The final concentration of nucleotide in the reaction mixture was 10 µM or 100 µM. (B and C) Far-UV CD spectra of Clp and the control protein GFP in the presence or absence of c-di-GMP as indicated.

FIG. 3. Modulation of the intracellular level of c-di-GMP changes the expression pattern of engXCA. (A) The effect of rpfG and clp mutations on engXCA transcription in Xcc. (B) Effect of c-di-GMP synthase (PA5487) and degradation enzyme (PA3947) on engXCA expression. The genes for in trans expression were cloned under the control of the lac promoter in expression vector pDSK519 (pDSK); the empty vector was introduced into wild-type strain...
8004 (WT) and its deletion mutant, ΔrpfG, as controls. (C) RT-PCR analysis of engXCA in the strains described above. The relative signal intensity for each strain was derived after normalization against the corresponding 16S rRNA loading control. In (A) and (B), the data are the means of three repeats and the error bars indicate standard deviation.

FIG. 4. The role of conserved amino acid residues implicated in cAMP binding in the functionality of Clp. (A) The effect of substituting the conserved amino acid residues on the regulatory activity of Clp in modulating engXCA expression. (B) The effect of substituting the conserved amino acid residues on the regulatory activity of Clp in modulating extracellular polysaccharide (EPS) production. The data are the means of three repeats and the error bars indicate standard deviation.

FIG. 5. The impact of amino acid substitution on the sensitivity of Clp to its inhibitory ligand c-di-GMP. (A) EMSA analysis of Clp and its derivatives (25 nM) in the presence or absence of c-di-GMP. (B) Effect of in trans expression of c-di-GMP synthase (PA5487) on engXCA expression in Xcc strains expressing wild-type Clp or substituted alleles. (C) The effect of in trans expression of c-di-GMP synthase (PA5487) on the virulence of Xcc strains expressing wild-type Clp or substituted alleles against cabbage. The experiment was repeated three times and the photograph, taken 4 days after inoculation, shows a representative set of results.
FIG. 6. The E99 residue of Clp may be a signature residue of c-di-GMP effectors with a cNMP binding domain. (A) Clp homologues can be grouped into two categories based on the signature amino acid E99. Solid arrows indicate the conserved residues corresponding to E99 and T149 of Clp. XCV0519, Xanthomonas campestris pv. vesicatoria (NCBI Accession No. CAJ22150); XAC0483, Xanthomonas axonopodis pv. citri (NCBI Accession No. AAM35374); XOO_3933, Xanthomonas oryzae pv. oryzae MAFF 311018 (NCBI Accession No. BAE70688); Clp, Xanthomonas campestris pv. campestris str. 8004 (NCBI Accession No. AAY47567); XfasM23_0792, Xylella fastidiosa M23 (NCBI Accession No. ACB92231); SmClp, Stenotrophomonas maltophilia K279a (NCBI Accession No. YP_001973974); Vfr, Pseudomonas aeruginosa PAO1 (NCBI Accession No. AAG04041); Avin_46100, Azotobacter vinelandii DJ (NCBI Accession No. AC080719); PSPTOT1_1546, Pseudomonas syringae pv. tomato T1 (NCBI Accession No. ZP_03395776); Crp, Escherichia coli str. K-12 substr. MG1655 (NCBI Accession No. AAC76382). (B) ITC analysis of c-di-GMP (500 µM) binding to SmClp. The dissociation constant ($K_d$) was estimated as $2.16 \pm 5.4 \times 10^{-7}$ M. (C) ITC analysis of SmClp binding cAMP (1 mM).

FIG. 7. Schematic representation of the role of Clp and c-di-GMP in the DSF-mediated QS regulation of virulence at (A) low and (B) high population density. The Clp-dependent virulence regulon is represented by vir, which includes engXCA and other virulence genes as depicted in our previous study (7).
Fig. 1

A

B

[Graph showing data with Molar Ratio on the x-axis and a peak and valley pattern on the y-axis.]

B

[Graph showing data with Molar Ratio on the x-axis and a linear trend on the y-axis.]
Fig. 2

A

B

C

Clp (nM) 0 50 200 200 200 200 200 200 200

Shift

Probe

Wavelength (nm)

Molar ellipticity (deg.cm$^2$.dmol$^{-1}$)

GST

GST + c-di-GMP

Wavelength (nm)

Molar ellipticity (deg.cm$^2$.dmol$^{-1}$)

Clp

Clp + c-di-GMP

Wavelength (nm)

Molar ellipticity (deg.cm$^2$.dmol$^{-1}$)

GST

GST + c-di-GMP
Fig. 3

A

B

C

16S
Signal
Intensity:
engXCA
WT      WT Δ rpfG
(pDSK)  (PA5487)    (pDSK)  (PA3947)     (clp)
100           12           18          50         127
Fig. 4

A

B

PengXCA-GUS (units)

EPS production (% Wt)

WT(pDSK)  
ΔnpF(pDSK)  
ΔnpG(pDSK)  
ΔnpFΔnpG(pDSK)  
ΔnpF(E86)  
ΔnpF(T148)  
ΔnpF(F86)
### Fig. 5

#### A

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#### B

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#### C

- **WT(pDSK)**
- **E99S(pDSK)**
- **T149S(pDSK)**
- **WT(PA5487)**
- **E99S(PA5487)**
- **T149S(PA5487)**
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### Figure 6

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Fig. 7

A

Membrane

Cytoplasm

RpfC

RpfF

RpfG

c-di-GMP

Clp

vir

B

Membrane

Cytoplasm

RpfC

RpfF

RpfG

c-di-GMP

P

Clp

Self-regulation

Virulence

DSF

GMP