The Pleiotropic Effects of c-di-GMP Content in *Pseudomonas syringae*

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ABSTRACT

Although the ubiquitous bacterial secondary messenger cyclic diguanylate (c-di-GMP) has important cellular functions in a wide range of bacteria, its function in the model plant pathogen Pseudomonas syringae (P. syringae) remains largely elusive. To this end, we overexpressed Escherichia coli diguanylate cyclase (YedQ) or phosphodiesterase (YhjH) in P. syringae, resulting in high or low in vivo levels of c-di-GMP. Via genome-wide RNA sequencing of these two strains, we found that c-di-GMP regulates (i) fliN, fliE, and flhA, which are associated with flagellar assembly; (ii) alg8 and alg44, which are related to the exopolysaccharide biosynthesis pathway; (iii) pvdE, pvdP, and pvsA, which are associated with the siderophore biosynthesis pathway; and (iv) sodA, which encodes a superoxide dismutase. In particular, we identified three promoters that are sensitive to elevated levels of c-di-GMP and inserted them into luciferase-based reporters that respond effectively to the c-di-GMP levels in P. syringae; these promoters could be useful in the measurement of in vivo levels of c-di-GMP in real time. Further phenotypic assays validated the RNA-seq results and confirmed the effect on c-di-GMP-associated pathways, such as repressing the type III secretion system (T3SS) and motility while inducing biofilm production, siderophore production, and oxidative stress resistance. Taken together, these results demonstrate that c-di-GMP regulates the virulence and stress response in P. syringae, which suggests that tuning its level could be a new strategy to protect plants from attacks by this pathogen.
The present work comprehensively analyzed the transcriptome and phenotypes that were regulated by c-di-GMP in *P. syringae*. Given that the majority of diguanylate cyclase and phosphodiesterase have not been characterized in *P. syringae*, this work provided a very useful database for the future study on regulatory mechanism (especially its relationship with T3SS) of c-di-GMP in *P. syringae*. In particular, we identified three promoters that were sensitive to elevated c-di-GMP level, and inserted them into luciferase-based reporters that effectively respond to intracellular level of c-di-GMP in *P. syringae*, which could be used as an economic and efficient way to measure relative c-di-GMP levels *in vivo* in the future.

**KEYWORDS**

c-di-GMP, pleiotropic effects, *Pseudomonas syringae*, T3SS
INTRODUCTION

The bacterial secondary messenger cyclic diguanylate (c-di-GMP) regulates multiple important functions, including transition from planktonic lifestyle to biofilm lifestyle and the biosynthesis of exopolysaccharides in the extracellular matrix of biofilms in many bacterial species (1-5). c-di-GMP is catalyzed by diguanylate cyclases (DGCs) and degraded by phosphodiesterases (PDEs). These enzymes are found in many bacterial species, such as Escherichia coli, Salmonella enterica, Bacillus subtilis, Pseudomonas aeruginosa (P. aeruginosa), and Clostridium difficile (4, 6-8).

In Pseudomonas syringae pv. tomato (Pto) DC3000, BifA and Chp8 have been identified as PDE and DGC, respectively (9, 10). The overexpression of BifA reduces the c-di-GMP level in vivo and elevates the virulence of pathogens in the Pseudomonas genus, whereas a ΔbifA strain shows an elevated c-di-GMP level and reduced necrosis and chlorotic symptoms during infections (10). Although BifA and Chp8 perform opposite functions, both affect virulence-related phenotypes such as motility and biofilm formation (9, 10). A high intracellular c-di-GMP level drastically inhibits the biosynthesis of flagellin and bacterial motility but enhances the formation of biofilm. In contrast, a low c-di-GMP level strengthens bacterial motility and attenuates biofilm production in many bacteria (2, 5, 11-14). In P. aeruginosa, c-di-GMP regulates bacterial motility by controlling the expression of FliA, the key regulator of flagellar synthesis (15).

A high intracellular level of c-di-GMP induces P. aeruginosa pyoverdine synthesis, which is dependent on exopolysaccharides and DGC (16-19). In iron-replete conditions, P. aeruginosa produces the major siderophore—pyoverdine—for the uptake of iron and rescue of iron starvation (20, 24). In P. syringae, pyoverdine is regulated by PvdS (20-22) and is assembled by non-ribosomal peptide synthetase (23). PvsA is also essential for the biosynthesis of pyoverdine in Pseudomonas fluorescens ATCC 17400 (24).

During infection in a host, c-di-GMP regulates bacterial resistance against oxidative stress, which is crucial for pathogens to survive the host immune response (25). For example, in Salmonella enterica, a low level of c-di-GMP decreases resistance against hydrogen peroxide (H₂O₂) (13). In P. aeruginosa, an increased c-di-GMP level
confers greater resistance to H$_2$O$_2$ (25). Scavenger-superoxide dismutases are expressed by pathogens to resist reactive oxygen species (ROS), which damage bacterial DNA, RNA, proteins, and the stability of cell membranes (26, 27). For instance, the superoxide dismutases SodA and SodB convert superoxide O$_2^-$ into H$_2$O$_2$, which is decomposed into O$_2$ and H$_2$O with the help of KatG, KatE, or the alkyl hydroperoxide reductase AhpC (28-30).

YedQ and YhjH, the DGC and PDE from *E. coli*, have been used to tune the c-di-GMP levels in *Burkholderia cenocepacia* (31), *Comamonas testosteroni* (32), *Pseudomonas aeruginosa* (17), *Pantoea alhagi* (33), and *Pseudomonas putida* (34) and to investigate the effects of c-di-GMP in these bacteria. Therefore, to explore the functions of c-di-GMP in *P. syringae*, we used plasmid pBBR1MCS to overexpress yedQ and yhjH in *P. syringae*. We hypothesized that c-di-GMP has a divergent effect on virulence and stress responses in *P. syringae*.

In this study, RNA-seq was performed to identify the c-di-GMP–dependent regulon in *P. syringae*. Based on RNA-seq results, luciferase-based reporters were constructed to efficiently measure the intracellular c-di-GMP level. Phenotypic assays were further used to demonstrate that c-di-GMP regulates many important biological pathways in *P. syringae*, such as regulation of the type III secretion system (T3SS), motility, biofilm production, siderophore production, and oxidative stress resistance.
Materials and Methods

Strains, plasmids, primers and growth conditions

The plasmids, bacterial strains, and primers used in this study are listed in Table 1. Unless otherwise indicated, the *Pseudomonas syringae* pv. *syringae* (*Psy*) B728a and its derivatives were grown in KB consisted of 20 g proteose peptone, 1.5 g anhydrous K$_2$HPO$_4$, 15 mL glycerol and 1.5 g MgSO$_4$ each liter with or without agar at 28 °C or shaking at 250 rpm. The concentration and categories of antibiotics were added as follows: for *Psy* B728a wild type (WT) strain, rifampicin 25 μg/mL in KB agar media; for over-expressing *yedQ* strain (OX-*yedQ*), supplemented with rifampicin 25 μg/mL and additional gentamicin 60 μg/mL in KB agar media but gentamicin 30 μg/mL in KB liquid media; for over-expressing *yhjH* strain (OX-*yhjH*) strain, supplemented with rifampicin 25 μg/mL and additional tetracycline 60 μg/mL; for these strains containing pMS402 plasmid, added 100 μg/mL kanamycin, while in *E. coli* it was added 50 μg/mL kanamycin. Experiments for the part of OX-*yedQ* and OX-*yhjH* construction, RNA-seq and liquid chromatography mass spectrometry (LCMS) quantification of c-di-GMP were performed at Nanyang Technological University in Singapore, other experiments were finished at Department of Biomedical Sciences, City University of Hong Kong.

Construction of c-di-GMP reporters

To report the c-di-GMP level in *P. syringae* sensitively, three c-di-GMP reporters of *Psy* B728a were constructed by inserting the promoters of Psyr_0610 (315 bp), Psyr_0685 (257 bp), Psyr_5026 (247 bp) to the promoter-less plasmid pMS402 (35), respectively (Table 1). Furthermore, to examine whether their homogenous genes in *Pto* DC3000 are also sensitive to c-di-GMP level in *Psy* B728a strain, we constructed the corresponding homogenous promoters originated from *Pto* DC3000 (Table 1). The constructed plasmids were electro-transformed into *Psy* B728a and its derivatives (they are the high c-di-GMP content strain OX-*yedQ* and low c-di-GMP level strain OX-*yhjH*) by using MicroPulser (Bio-Rad) with 1.8 kv measurement during every electroporation. OX-*yedQ* strain and OX-*yhjH* strain were constructed with the carrying of pBBR1MCS-5 plasmid and pBBR1MCS-3 plasmid, respectively. Positive reporter strains were cultured at mid-log growth phase (OD$_{600}$ = 0.6). Luminescence
value (counts per second, cps) of bacteria was recorded using a 96-well white but opaque microplate in Biotek microplate reader with luminescence fiber optics type. The optical density of bacteria in each well was determined immediately using a 96-well transparent bottom cell culture plate of Fisher Scientific microplate reader at 600 nm.

**RNA-seq analysis**

To test the effect of high c-di-GMP level in *Psy B728a* on the transcriptome, cells were recovered and cultured to early stationary (*OD$_{600}$ ~ 2; no addition of antibiotics) in NYGB medium consisting of 8 g nutrient broth, 10 g glucose and 5 g yeast extract each liter. Cells were mixed with bacterial RNA Protect Reagent (Qiagen) to keep intact RNA. Total RNA was purified using RNeasy Mini Kit (Qiagen). RNase-free DNase Set (Qiagen) was applied to remove DNA contaminant through on-column DNase digestion. Second DNA removal step was applied using Ambion™ TURBO DNA-free Kit. rRNA was depleted using Ribo-Zero rRNA removal Kit (Illumina).

The integrity and concentration of total RNA and DNA contamination were examined by Agilent TapeStation System (Agilent Technologies) and Qubit® 2.0 Fluorometer (Invitrogen). Reverse transcription to cDNA was done using NEBNext RNA first and second strand synthesis module (NEB). Analysis of gene expression was carried out via Illumina RNA-Seq. Libraries were produced using an Illumina TruSeq Stranded messenger RNA Sample Prep Kit. The libraries were sequenced using Illumina HiSeq 2500 platform with a paired-end protocol and read lengths of 101 nt. The raw sequence data were streamlined using Trim Galore v0.4.5 software (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to truncate or filter reads of low quality (parameter: --paired -q 20 --phred33 --illumina --length 36). High quality reads were then aligned to the *Psy B728a* reference genome (GenBank accession: NC_007005.1) and annotation file (ASM1224v1) using Tophat v2.2.6 (36) with parameter “-N 2 -g 1”. Only the reads mapped once were considered. From the resulting alignments, SAMtools v1.6 (37) were applied to sort the bam file. The differentially expressed genes were identified by performing Cuffdiff v2.2.1 (38) with p value smaller than 1e-5. Each sample in RNA-seq assay was repeated three times. The RNA-seq datasets have been deposited in National Center for Biotechnology Information (NCBI) with an accession number GSE120889.
**Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)**

RT-qPCR was done using OX-yedQ and OX-yhjH strain to validate the RNA-seq data. Briefly, 500 µL fresh and overnight bacterial cells (OD$_{600}$ = 1) were harvested by centrifugation at 2400 × g for 5 min at 4 °C. The extraction of total RNA followed the manufacturer’s instructions of total RNA Purification kit (Sangon Biotech). cDNA was synthesized by TIANScript RT Kit (TIANGEN). RT-qPCR used SuperReal PreMix Plus (TIANGEN). The mass of cDNA is 70 ng per reaction and 16S rRNA were selected as the internal reference. Key genes involved in T3SS and biofilm were analyzed. mRNA expression was evaluated for each sample using the cycle threshold (Ct) value. Relative gene expression was calculated as follows: ΔCt=ΔCt$_{\text{target}}$-ΔCt$_{16s}$. The fold-change for the treatment was defined as the relative expression compared with the control group, and was calculated as follows: $2^{-\Delta\Delta C_{t}}$, where $\Delta\Delta C_{t} = C_{t_{\text{control}}} - C_{t_{\text{target}}}$. The error bar was calculated through SD value. The experiment was repeated twice.

**Quantification of c-di-GMP extracted from Psy B728a using liquid chromatography mass spectroscopy (LCMS)**

Psy B728a cells were recovered on KB agar plate. Single colonies were picked and grew overnight in KB medium with respective antibiotics for strains carrying plasmid (30 µg/mL tetracycline and 30 µg/mL gentamicin) and no antibiotics for wild-type at 28 °C, 200 rpm. Overnight culture were diluted to OD$_{600}$ ~ 0.01 in KB medium and grew for 16 hours to stationary phase (OD$_{600}$ ~ 3 for WT and 1.3 ~ 1.6 for two mutants). Biological triplicates were used for each strain. 5 mL of cells were collected from each sample and washed with 1 mM ammonium acetate. c-di-GMP was extracted using lysis buffer consisting of ammonium acetate/methanol/water in 40%/40%/20% ratio. Sonication using probe sonicator was done using 40% amplitude for one and half minute in 10 sec ON/10 sec OFF cycle to give better cell lysis. Cell debris was removed by centrifuge and supernatants containing c-di-GMP were dried to remove lysis buffer using Labconco SpeedVac concentrator. All processing steps were performed on ice or at 4 °C. Dried samples were reconstituted in 200 µL of LCMS grade water and injected into LCMS directly with injection volume of 5 µL. Waters BEH C18 column (1.7 µm, 2.1x50 mm) was used for High Performance Liquid Chromatography (HPLC). Gradient run were applied using water containing
10 mM ammonium formate, 0.1% formic acid as mobile phase A and methanol containing 0.1% formic acid as mobile phase B. Mobile phase A gradient of 100% at 1 min; 80% at 3 min; 60% from 4 to 4.5 min; 100% from 4.6 to 6 min were applied at flow rate of 0.3 mL/min. Xevo TQ-S (Waters) mass spectrometer was used to identify c-di-GMP with reference to c-di-GMP standard (Sigma) using ESI positive mode at 40 V collision energy. Three technical replicates were measured for each sample. c-di-GMP was detected at retention time of 1.74 min with MRM transition to 151.0 and 135.0. Quantities of c-di-GMP were calculated and normalized against protein level and illustrated in the bar chart. c-di-GMP level in OX-yhjH samples were undetectable.

Protein extraction was done for normalizing c-di-GMP concentrations among samples. 1 mL of bacterial culture was collected from each sample at the beginning of extraction from the same sample tubes. Cell pellet was collected and re-suspended in 1 mL of 0.1 M NaOH. Cells were heated at 95 °C for 10 min. Total protein of each sample was measured using Qubit® protein assay kit (Invitrogen).

**Gene ontology (GO) and KEGG pathway enrichment analysis**

GO enrichment analysis and KEGG pathway enrichment analysis of DEGs was performed using DAVID 6.8 online analysis (https://david.ncifcrf.gov/gene2gene.jsp). The GO and KEGG term with p value < 0.05 was defined as significantly enriched term.

**Congo red assay and biofilm formation assay**

Congo red agar plate assay was carried out according to the previous study (24) with minor changes to compare the production of exopolysaccharide between elevated c-di-GMP level strain OX-yedQ and OX-yhjH. Germ filtered Congo red dye (100 µg/µL) was added to KB medium with 1.5% agar. 5 µL liquid overnight bacteria culture was spotted to the Congo red plate (with rifampicin 25 µg/mL) and cultured at 28 °C. The colony staining was photographed after 3 days. The experiment was repeated with two independent bacterial cultures (3 plates for each strain).

Biofilm detection experiment was performed in minor modifications as reported previously (41). In brief, overnight bacterial culture was transferred to a 10 mL
borosilicate tube containing 2 mL KB medium (without antibiotics) with OD$_{600} = 0.1$ and cultured statically at 28 ℃ for 3-4 days. 0.1% crystal violet was used to stain those biofilm cells adhered to the tube tightly for 15 min and other cells bound to tube loosely was washed off with distilled deionized water (ddH$_2$O). Tubes were washed for three times with ddH$_2$O gently, and the remaining crystal violet was fully dissolved in 250 µL 95% ethanol with constantly shaking after photograph. 200 µL of this eluate was transferred to a clear and transparent 96-well plate to measure its optical density at 590 nm using Biotek microplate reader. Three tubes were used for each strain, and the experiment was repeated with three independent bacterial cultures.

**Measurement of H$_2$O$_2$ resistance**

For growth assay, overnight bacterial culture (OX-yeDQ and OX-yhjH; OD$_{600} = 1.0$) was diluted to OD$_{600} \sim 0.01$ with KB media (without antibiotics) with or without 2 mM H$_2$O$_2$. Two hundred µL diluted culture media was added into a sterile 96-well plate (Thermo Fisher Scientific) at 28 ℃. After 12 h, 24 h, 36 h and 42 h, bacterial growth were recorded by using a Biotek microplate reader at 600 nm. The experiment was repeated with two independent bacterial cultures, and 3 replicates were used for each strain. As described previously (42), in the hydrogen peroxide resistance colony assay, bacterial culture was adjusted to OD$_{600} = 1.0$. KB agar plates were supplemented with 0.5 mM H$_2$O$_2$, and 10 µL of serially-diluted bacterial cultures were incubated on the plates at 28 ℃ for 3 days. Three plates were used for two strains, and the experiment was repeated with three independent bacterial cultures.

**CAS agar assay for iron uptake**

Chrome azurol S (CAS) agar assay was carried out as previously described (23). To make 100 mL CAS stock solution, 60.5 mg CAS powder (Sigma) was added to 50 mL ddH$_2$O, followed by 10 mL 1 mM FeCl$_3$. Then 72.9 mg hexadecyltrimethylammonium bromide (HDTMA) (Sigma) was fully dissolved in 40 mL ddH$_2$O. At last, the entire HDTMA solution (40 mL) was slowly poured into 60 mL CAS solution with constantly shaking to form 100 mL CAS stock solution. CAS agar plate was prepared by 9 parts freshly autoclaved 1.5% agar KB plate and 1 part CAS stock solution. After agar solidified, two circular holes were dug into a CAS agar by the round end of a 1 ml sterile pipette tip. About 2 µL overnight bacterial
culture (OX-\textit{yedQ} and OX-\textit{yhjH}; \text{OD}_{600} = 1.0) of experiment group and control group was added into one of two holes respectively and cultivated at 28 °C. The CAS plate (without antibiotics) was photographed after 3 days against a white background. Three CAS plates were used for two strains, and the experiment was repeated with three independent bacterial cultures.

**Motility assay**

The method of motility experiment was performed based on the previous study (41) with minor modifications. Swimming and swarming ability were tested on KB plate with 0.3% and 0.4% agar (MP Biomedicals) respectively. Overnight KB cultures were inoculated with adjusting the same bacterial density (OX-\textit{yedQ} and OX-\textit{yhjH}; \text{OD}_{600} = 1.0). 2 µL aliquots were dotted on swimming and swarming plates and cultured at 28 °C. The quantitative analysis was detected by the diameter of motility zone. Photographs were taken after 36 h for swimming plate and 72 h for swarming plate. Three motility plates were used for two strains, and the experiment was repeated with three independent bacterial cultures.

**Statistical analysis**

Data are presented as mean ± standard error. Data were tested for normality and analyzed using the unpaired Student’s \( t \)-test and one asterisk * indicates significant difference with \( p < 0.05 \). Each experiment was performed three times with similar results. ** indicates significantly difference with \( p < 0.01 \), *** indicates significantly difference with \( p < 0.001 \).
RESULTS

Overexpression of YedQ increases the intracellular c-di-GMP level in *P. syringae*

To modulate the intracellular c-di-GMP level in *P. syringae*, OX-yeQ and OX-yhjH were generated via the overexpression of YedQ or YhjH, which function as c-di-GMP synthase or phosphodiesterase in *Psy* B728a, respectively. The c-di-GMP concentrations of wild-type, OX-yeQ, and OX-yhjH strains were quantified by LCMS at the stationary growth phase. As expected, the c-di-GMP concentration in OX-yeQ (102.12 pmol/mg) was more than 20 times greater than that in the wild type (4.91 pmol/mg) (p = 0.00026) (Figure 1), which demonstrates that the overexpression of OX-yeQ leads to the elevated production of intracellular c-di-GMP in *P. syringae*. In contrast, the level of c-di-GMP in the OX-yhjH strain was below the detection limit of LCMS (Figure 1), which suggests that overexpression of YhjH results in the degradation of c-di-GMP in *P. syringae*. Therefore, the heterogeneous expression of YedQ in *P. syringae* is effective in generating a high level of c-di-GMP.

c-di-GMP-dependent regulon in *P. syringae*

To reveal the c-di-GMP-dependent regulons in *P. syringae*, RNA-seq was performed to compare the transcriptomic profiles of the OX-yeQ and OX-yhjH strains; 818 differentially expressed genes (DEGs) were identified between these two strains (Figure 2A and Table S1). Of these DEGs, 352 were up-regulated and 466 were down-regulated in the OX-yeQ strain (p < 0.05; more than 2-fold enrichment; Figure 2A). Enrichment analyses were performed on these DEGs via GO or the KEGG pathway. GO enrichment analysis highlighted the genes involved in flagellum-dependent cell motility, phosphorelay signal transduction, flagellum organization, siderophore transport, cell adhesion, and chemotaxis (Figure 2B). Notably, the motility-related genes were significantly enriched (Figure 2B). The genes associated with oxidoreductase activity and siderophore biosynthesis and transport were also enriched significantly (p < 0.05; Figure 2B). According to KEGG analysis, genes associated with two-component systems were identified as the most enriched pathway (Figure 2C). Flagellar assembly, fatty acid biosynthesis, and bacterial chemotaxis were also significantly enriched (Figure 2C). In sum, transcriptomic profiling analysis characterized the c-di-GMP-dependent regulon in *P. syringae*, thus indicating that c-
di-GMP regulates multiple biological pathways, including flagellar motility, iron-binding siderophore, chemotaxis, and oxidoreductase activity. Furthermore, the RNA-seq assay was repeated by using the WT and OX-yedQ strain (Table S2). GO enrichment analysis highlighted the genes involved in flagellum-dependent cell motility, phosphorelay signal transduction, and bacterial chemotaxis (Table S3), which is consistent with the RNA-seq assay by using OX-yedQ and OX-yhjH strains (Figure 2 and Table S1).

**Luciferase-based reporters reflect the c-di-GMP level in vivo**

Mass spectrometry is widely used for the accurate quantification of the intracellular c-di-GMP concentration (43, 44). Here we attempted to develop an economical and convenient method to measure the relative c-di-GMP concentrations in *P. syringae* using transcriptional fusion bioassays. We sought to make c-di-GMP biological reporters by fusing the promoters of c-di-GMP-induced genes to a promoter-less luciferase gene. We first selected three top-induced candidates (Psyr_0610, Psyr_0685, and Psyr_5026) from our RNA-seq datasets (Figure 3A). These three constructed plasmids were electro-transformed to OX-yedQ and OX-yhjH to measure the luminescence value (counts per second). All of the reporters (Psyr_0610, Psyr_0685, and Psyr_5026) showed significantly higher lux levels in OX-yedQ than in OX-yhjH (100-, 60-, and 20-fold, respectively; Figure 3B), which indicates that these three promoters were very sensitive to the high levels of c-di-GMP in *Psy* B728a. In particular, the Psyr_0610 promoter was the most sensitive, with an induction of 100-fold, so it could be used as an efficient method to measure in vivo c-di-GMP levels (Figure 3B).

To examine whether their homogenous genes in *Pto* DC3000 were also sensitive to the c-di-GMP level, we constructed their counterparts in *Pto* DC3000 (PSPTO_0704, PSPTO_3756, and PSPTO_5471). The resulting plasmids were electro-transformed into OX-yedQ and OX-yhjH and showed even better results than their counterparts in *Psy* B728a. These promoters were induced ~1500-, 13-, and 500-fold, respectively, in OX-yedQ (Figure 3C). This luciferase-based assay also confirmed that overexpression of YedQ elevated the production of c-di-GMP in *P. syringae*. In sum, our newly constructed luciferase-based reporters can be used for the sensitive monitoring of c-di-GMP levels in vivo.
Expression of T3SS is suppressed by c-di-GMP

Studies have shown that c-di-GMP can modulate virulence and T3SS in some plant pathogens (4, 8, 44). For example, a higher level of c-di-GMP leads to the repression of T3SS in *P. aeruginosa* (44). To explore the effects of c-di-GMP in *P. syringae* T3SS and virulence, we identified 12 T3SS genes from our RNA-seq data and verified them by using RT-qPCR. As shown in Figure 4, the expression of *hrpR*, *hrpL*, *hrpA2*, *hrpB*, *hrpF*, *hrcC*, *hrcN*, *hrcR*, *avrB3*, *avrE1*, and *avrRPM1* was suppressed by 2- to 3-fold in the OX-*yedQ* strain in minimal medium compared with that of OX-*yhjH*, thus suggesting an inhibitory effect of c-di-GMP on T3SS, which is consistent with the result for *P. aeruginosa* (44). We also tested whether the inhibition was mediated by RhpR, a known T3SS repressor (45, 46). However, the expression of *rhpR* showed no significant difference between OX-*yedQ* and OX-*yhjH* (Figure 4), which suggests that c-di-GMP inhibits T3SS via factors other than RhpRS.

c-di-GMP negatively controls motility by regulating the expression of *flhA*, *fliN*, and *fliE*

In other *Pseudomonas* species, motility was inhibited by higher intracellular c-di-GMP level strains (5, 12, 14). Our RNA-seq results showed that seven known operons (*flhAF*, *fliLMNPOQR-flhB*, *fliEFJ*, *fliS-3462*, *flgFGHIJKL*, *flgBCDE*, and *flgA*) associated with flagellar synthesis were significantly down-regulated in the OX-*yedQ* strain compared with the OX-*yhjH* strain (Table S1; Figure 5A). In addition, the transcription levels of a group of hypothetical genes, such as *Psyr_3466* (encoding flagellin; 4-fold less) and *Psyr_3460* (encoding flagellar sensor histidine kinase FleS), were also repressed (12-fold less) by c-di-GMP (Table S1). The phenotypic experiments showed that the swarming motility and swimming motility of OX-*yedQ* were significantly compromised (3-fold less) when compared with those of OX-*yhjH* (Figure 5B and 5C). The elevated c-di-GMP level had significant inhibitory effects on the motility of *P. syringae*.

c-di-GMP is required for biofilm formation in *P. syringae*

In *P. aeruginosa*, the overexpressing YhjH strain showed less biofilm formation than the wild type (20). In *E. coli*, c-di-GMP positively regulates genes associated with motile–sessile transition and biosynthesis and with the secretion of
exopolysaccharides (EPS) in biofilms (3, 4). Here, we speculated that c-di-GMP has similar functions in *P. syringae*. As expected, the expression of *alg8* and *alg44*, which are involved in EPS biosynthesis, was increased by 3- to 4-fold in OX-*yedQ* based on RT-qPCR (Figure 6A). To further verify this regulation, the Congo red assay and quantitative detection of biofilms were performed in both strains. As shown in Figure 6B, the EPS production was greater in the OX-*yedQ* strain (more mucoid colonies) than in the OX-*yhjH* strain. Biofilm formation was also much higher in the OX-*yedQ* strain, as shown in Figures 6C and 6D. These results indicate that c-di-GMP positively regulates biofilm formation in *P. syringae*.

**c-di-GMP positively regulates siderophore production**

Of the DEGs in our RNA-seq data, genes catalogued as “iron ion binding” or as “siderophore transport” were significantly enriched in gene ontology analysis (Figure 2B). The expression levels of pyoverdine transporter PvdE (32) and peptide synthase PvdP and PvsA (30, 31) were up-regulated 40-, 7-, and 20-fold, respectively (Figure 7A). Given that pyoverdine was characterized by siderophore production, iron acquisition, virulence, and growth in iron-restricted conditions (23), we further tested whether a high c-di-GMP content could induce siderophore production in *P. syringae*. In a CAS-based iron uptake assay, the OX-*yedQ* strain exhibited a larger orange halo (more than 2-fold) than OX-*yhjH* (Figure 7B), which is consistent with a previous study in *P. syringae pv. phaseolicola* 1448A (23). c-di-GMP positively regulates the biosynthesis, assembly, and transport of siderophore in *P. syringae*.

**c-di-GMP positively regulates resistance to oxidative stress**

Cytotoxic ROS, such as the superoxide radicals O$_2^-$, H$_2$O$_2$, and hydroxyl radicals, can damage DNA, proteins, and lipids, resulting in a toxic effect on pathogenic bacteria (26). To protect bacteria from the ROS produced by plant cells during infection, it is important for pathogenic bacteria to inactivate ROS with their antioxidant enzymes such as superoxide dismutase. Based on our RNA-seq data, genes that encode for superoxide dismutase, such as *sodA*, were differentially expressed. Notably, the RNA-seq data showed that the expression of *sodA* was induced about 40-fold in OX-*yedQ*, while the expression of *sodB* and *sodC* was repressed (Figure 8A). We hypothesized that c-di-GMP mediates the resistance to oxidative stress in *P. syringae*. To test the
phenotypes of the elevated c-di-GMP level, H$_2$O$_2$ tolerance tests were performed in liquid and solid KB media for OX-yhjH and OX-yedQ strains. OX-yhjH showed no growth in 6 mM H$_2$O$_2$, but OX-yedQ grew well in 10 mM H$_2$O$_2$ for 18 h (data not shown). OX-yedQ grew better in 2 mM H$_2$O$_2$ than OX-yhjH (Figure 8B). The result of plate assay of H$_2$O$_2$ resistance also demonstrated that the OX-yedQ strain showed stronger tolerance against H$_2$O$_2$ than OX-yhjH (Figure 8C). Taken together, the results suggest that c-di-GMP positively regulates resistance to oxidative stress by inducing the transcription of sodA.
DISCUSSION

The overexpression of *E. coli* YedQ and YhjH protein is widely used to study the function of c-di-GMP in many *Pseudomonas* species (47, 48). The results of mass spectroscopy confirmed that the exogenous YedQ and YhjH enabled *P. syringae* to alter the c-di-GMP level. The concentration of c-di-GMP was below the detection limit in the OX-yhjH strain. The three most sensitive c-di-GMP–responsive reporters (Psyr_0610-lux, Psyr_0685-lux, and Psyr_5026-lux) were identified and constructed and can be used to measure c-di-GMP levels in vivo. We were intrigued to find that the promoter of exogenous gene PSPTO_0704 of *Pto* DC3000 showed the highest induction level (1000-fold) between the two strains. In *P. aeruginosa*, the c-di-GMP–responsive reporter brlR-lux was induced 100-fold by a high c-di-GMP level (49). The differences in the fold changes between PSPTO_0704-lux and brlR-lux may have been caused by the differences in the intracellular c-di-GMP levels in the two *Pseudomonas* strains.

The downregulation of a group of T3SS genes, including *hrpR, hrpA2, hrpB, hrpF, hrpL, hrcC, hrcN, hrcR, avrB3, avrE1*, and *avrRPM1* in strain OX-yedQ, with a high c-di-GMP level, suggests that c-di-GMP negatively regulates T3SS in *P. syringae*. However, the underlying mechanism requires further study. Studies have shown that c-di-GMP inhibits T3SS but activates T6SS in different bacteria (44, 50, 51). Similarly, T3SS genes were regulated by c-di-GMP in *P. syringae* (Table S1).

Jenal et al. showed that c-di-GMP regulates bacterial lifestyles, including swimming, swarming, and biofilm formation, to alter bacterial virulence in *P. aeruginosa* (4). Chp8 elevates EPS production and negatively regulates swarming motility, whereas BifA negatively regulates swarming motility and positively regulates swimming motility in *Pto* DC3000 (9, 10). In *P. aeruginosa* PA14 and *P. putida* KT2442, BifA only affects swarming motility (52). In this study, an elevated level of c-di-GMP suppressed both swimming and swarming motility in *P. syringae* (Figure 5B and 5C). The differences between Psy B728a and other *Pseudomonas* strains may have resulted from other functions of Chp8 and BifA. Biofilm production was positively regulated by c-di-GMP in *P. syringae*, which is consistent with the results for other bacterial species (4, 8). Furthermore, our study showed that c-di-GMP elevated the expression of LPS-related genes. The expression of Psyr_0610 (encoding O-antigen ABC
transporter, permease protein) and Psyr_0612 (encoding lipopolysaccharide biosynthesis protein) was 100-fold higher in the OX-yedQ strain than in OX-yhjH, which suggests that c-di-GMP regulates biofilm formation by elevating the production of LPS in *P. syringae*.

Pyoverdine production and oxidative stress resistance are regulated by c-di-GMP in *P. syringae* (25, 53). *Pseudomonas* species synthesize pyoverdine, the key virulent factor, to uptake iron from the extracellular medium to rescue iron starvation (53). c-di-GMP positively controls the siderophore pyoverdine to acquire iron from iron-replete medium (16). In addition to *pvdE*, *pvdP*, and *pvsA*, five genes that encode the putative siderophore non-ribosomal peptide synthase (Psyr_1956, Psyr_1957, Psyr_1958, Psyr_1959, and Psyr_1960) were significantly up-regulated in strains that overexpressed c-di-GMP (Table S1), which suggests that c-di-GMP regulates the uptake of iron by inducing the synthesis, export, and assembly of pyoverdine in *P. syringae*. c-di-GMP regulates the oxidative stress resistance in many bacterial strains (13, 28, 54), but the mechanism remains largely unclear. These results indicate that SodA, but not SodB or SodC, plays a major role in the c-di-GMP-mediated response against oxidative stress.

Taken together, these results demonstrate that the pleiotropic molecule c-di-GMP globally regulates many important intracellular activities and behaviors in *P. syringae*. In particular, c-di-GMP inhibits motility and T3SS and induces biofilm formation, pyoverdine production, and oxidative stress resistance in *P. syringae*. The newly constructed *P. syringae*-specific lux-reporters provide an economical and effective method to detect c-di-GMP levels *in vivo*. We propose that tuning the c-di-GMP level offers a new strategy to protect plants from attacks by *P. syringae*. 


AUTHOR CONTRIBUTIONS

TW, LY and XD designed the study and wrote the paper. TW and ZC performed experiments, analyzed data and generated figures. XL and CH edited the paper. WZ performed RNA-seq analysis. YX helped to construct plasmids. YZ helped to measure lux-luminescence. All authors reviewed the results and supported the final version of the manuscript.

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**FIGURE LEGENDS**

**Figure 1.** The quantification of intracellular c-di-GMP concentration by mass spectroscopy

Quantification in high c-di-GMP content strain OX-yedQ, low c-di-GMP strain OX-yhjH and Psy B728a WT by mass spectroscopy analysis. nd, not determined. * stands p value < 0.05, *** indicates p value < 0.001. by Student unpaired two-tailed t test compared to Psy B728a WT with equal variance. OX-yhjH strain was under the lowest detection value. The experiment was repeated with three independent bacterial cultures.

**Figure 2.** RNA-seq analysis between OX-yedQ and OX-yhjH strains

(A) Pie chart of 818 DEGs significantly regulated by high level of c-di-GMP. The number of down-regulated DEGs is indicated by orange; the number of up-regulated DEGs is indicated by blue. All experiments were performed in triplicate. Error bars indicate standard deviations. (B) Gene Ontology enrichment analysis. Gene analysis in “Biological Process”, “Cellular Component” and “Molecular Function” category for genes up-regulated and down-regulated in response to elevated c-di-GMP level. In all, GO terms were over-represented by > 2-fold enrichment values, with p value < 0.05. (C) Functional classification of KEGG pathway. The KEGG pathways were summarized in seven main categories for up-regulated and down-regulated genes: two-component system, flagellar assembly, bacterial chemotaxis, fatty acid metabolism, biotin metabolism, starch and sucrose metabolism, tryptophan metabolism. The x-axis indicated the numbers of genes of the KEGG metabolic pathways. The y-axis indicated terms of KEGG metabolic pathways including two items of up regulation and five items of down regulation. In all the categories the p value was < 0.05.

**Figure 3.** Five c-di-GMP-sensitive genes in *P. syringae*

(A) Fold-change for five genes based on the RNA-seq results. OX-yedQ is the strain harboring pBBR1MCS5-yedQ. (B) Luminescence of strains with pMS402-lux reporters driven by promoters of Psyr_0610, Psyr_0685 and Psyr_5026. (C) Luminescence of strains with pMS402-lux reporters driven by promoters of
Figure 4. c-di-GMP negatively regulated T3SS in P. syringae

RT-qPCR of genes related to T3SS. The relative gene expression level in the OX-yhjH was set to 1, and the other values were adjusted accordingly. *, significantly different between OX-yedQ and OX-yhjH; p < 0.05. **, significantly different between OX-yedQ and OX-yhjH; p < 0.01. ***, significantly different between OX-yedQ and OX-yhjH; p < 0.001. All experiments were performed in duplicate. Error bars indicate standard deviations.

Figure 5. Motility is negatively regulated by c-di-GMP in P. syringae

(A) Fold-change from RNA-seq result of genes related to flagellar motility. The relative gene expression level in the OX-yhjH was set to 1, and the other values were adjusted accordingly. (B) Swimming agar plate assay. Photos were taken after 36 h at 28 °C. The swimming ability of different strains was determined by the diameter of zone of motility as the bar chart shows. (C) Swarming agar plate assay. Phenotypic photos were taken after 72 h at 28 °C. The swarming ability of different strains was determined by the diameter of zone of motility as the column shows. * indicates significantly difference between OX-yedQ and OX-yhjH, p < 0.05. ** indicates p < 0.01. *** p < 0.001.

Figure 6. High c-di-GMP enhanced biofilm production

(A) RT-qPCR validation of alg8, alg44 for OX-yedQ compared to OX-yhjH. The relative gene expression level in the OX-yhjH was set to 1, and the other values were adjusted accordingly. (B) Crystal violet staining of OX-yedQ and OX-yhjH for quantifying relative biofilm biomass. The absorbance of crystal violet at 590 nm was measured. Photo in the upper part illustrates biofilm formed at liquid-air interface by the two strains and stained by crystal violet in tubes. (C) Congo red phenotypic experiment of OX-yedQ and OX-yhjH. Photos were taken after 4 days of cultivation at 28 °C. **, significantly different between OX-yedQ and OX-yhjH; p < 0.01. ***, significantly different from OX-yedQ at 12h time point; p < 0.05. **, significantly different from OX-yhjH at 12h time point; p < 0.01. ***, significantly different from OX-yhjH at 12h time point; p < 0.001. All experiments were performed in triplicate. Error bars indicate standard deviations.
significantly different between OX-yedQ and OX-yhjH; p < 0.001. Three Congo red plates were used for two strains, and the experiment was repeated with three independent bacterial cultures. Error bars indicate standard deviations.

Figure 7. OX-yedQ strain produced excess pyoverdine

(A) The expression fold-change of pvdP, pvdE and pvsA based on RNA-seq data. The relative gene expression level in the OX-yhjH was set to 1, and the other values were adjusted accordingly. (B) CAS agar plate experiment. The CAS plate was photographed after 48 h of cultivation with white background. **, significantly different between OX-yedQ and OX-yhjH; p < 0.01. ***, significantly different between OX-yedQ and OX-yhjH; p < 0.001. Three CAS plates were used for two strains, and the experiment was repeated with three independent bacterial cultures. Error bars indicate standard deviations.

Figure 8. OX-yedQ strain was more resistant to H₂O₂ in P. syringae

(A) The expression of sodA, sodB and sodC based on RNA-seq. The relative gene expression level in the OX-yhjH was set to 1, and the other values were adjusted accordingly. (B) OD₆₀₀ of bacterial culture in 2 mM H₂O₂ in KB liquid medium after 48 h of cultivation. The OD₆₀₀ was detected for every 12 hours. **, significantly different between OX-yedQ and OX-yhjH; p < 0.01. ***, significantly different between OX-yedQ and OX-yhjH; p < 0.001. All experiments were performed in triplicate. Error bars indicate standard deviations. (C) The hydrogen peroxide resistance of colony assay. Bacterial culture was adjusted to OD₆₀₀=1.0. KB agar plate was added 0.5 mM H₂O₂ and was dotted 10 µL of 10 folds-diluted bacterial. Photos were taken after 3 days of cultivation at 28 °C. Three H₂O₂ plates were used for two strains, and the experiment was repeated with two independent bacterial cultures.

Figure 9. Schematic of pleiotropic effects of c-di-GMP in P. syringae

Over-expressed YedQ increased the c-di-GMP content in P. syringae. Enhanced c-di-GMP level positively controlled biofilm formation, oxidative stress resistance and siderophore production, but negatively regulated motility and T3SS. Enhanced c-di-GMP also regulated ABC transporters, two-component systems, oxidative
phosphorylation and fatty acid metabolism. Solid black arrows indicate positive regulation, solid line T-bars present negative regulation, dashed line presents direct or indirect influence.
Table 1. Strains, plasmids and primers used in this study

| Strain, plasmids and primers | Features or sequence(5’-3’) | Reference or Application |
|-----------------------------|-----------------------------|--------------------------|
| **Strains**                 |                             |                          |
| *Pseudomonas syringae* pv.  |                             |                          |
| syringae B728a              | Prototypic wild-type strain; rif<sup>+</sup> | (55)                     |
| OX-yedQ                    | *Psy* B728a contains pBBR1MCS5-yedQ; rif<sup>+</sup> | This study              |
| OX-yhjH                    | *Psy* B728a contains pBBR1MCS3-yhjH; rif<sup>+</sup> | This study              |
| **Plasmids**               |                             |                          |
| pBBR1MCS-5                 | Overexpression vector; Gm<sup>+</sup> | (56)                     |
| pBBR1MCS-3                 | Overexpression vector; Tc<sup>+</sup> | (63)                     |
| pBBR1MCS5-yedQ             | Overexpresses YedQ under the lac promoter (<i>HindIII/BamHI</i>), the <i>yedQ</i> gene is cloned from the pYedQ plasmid vector; Gm<sup>+</sup> | (57)                     |
| pBBR1MCS3-yhjH             | Overexpresses YhjH under the lac promoter (<i>HindIII/BamHI</i>), the <i>yhjH</i> gene is cloned from the pYhjH plasmid vector; Gm<sup>+</sup> | (48)                     |
| pMS402                     | Expression reporter plasmid carrying the promoterless luxCDABE; Kn<sup>+</sup> | (58)                     |
| pMS402-0610                | <i>lux</i>-reporter fused with the promoter of Psyr_0610; Kn<sup>+</sup> | This study              |
| pMS402-0685                | <i>lux</i>-reporter fused with the promoter of Psyr_0685; Kn<sup>+</sup> | This study              |
| pMS402-1131                | <i>lux</i>-reporter fused with the promoter of PSPTO_1131; Kn<sup>+</sup> | This study              |
| pMS402-3767                | <i>lux</i>-reporter fused with the promoter of PSPTO_3767; Kn<sup>+</sup> | This study              |
| pMS402-5026                | <i>lux</i>-reporter fused with the promoter of PSPTO_5026; Kn<sup>+</sup> | This study              |
| **Primers names**          |                             |                          |
| Psyr0610BamHI-F            | TCGTCTTCACCTCGAGGGGATCCGAG GAGCCTCGCTTGTCAAG | Reporter construction |
| Psyr0610BamHI-R            | GCGGCGCGCACTAGAGGATCCTAATG AGAAAATCAGAGAG | Reporter construction |
| Psyr0685BamHI-F            | TCGTCTTCACCGCGGAGGGATCCTCA TCGCTCTTCGTGTTG | Reporter construction |
| Psyr0685BamHI-R            | GCGGCGCGCACTAGAGGATCCTGACAT | Reporter construction |
GCCGTCCTTGCGTC
Psyr5026BamHI-F
TCGTCTTCACTCGAGGGGATCCCAC
CCTGTTTGTCGCCCTCG
Psyr5026BamHI-R
GCCGCCCCAAGGTAGGATCCCTCAG
GATTTCCCAAGAGT
hopAH2-F
AGGACCTGAAAGCGATTGGA
CCTGTTGTGCGCCTCG
Reporter
construction
hopAH2-R
GAGCTTATCAAACGGTCCGC
for RT-qPCR
validation
AvrE1-F
CATAGCAACTCCACAGCGAC
for RT-qPCR
validation
AvrE1-R
TCATCAATGGTCACGTTCGC
for RT-qPCR
validation
HrpA2-F
CAGGGCATCAACAGCGTA
for RT-qPCR
validation
HrpA2-R
GTCGATACTGTACGTCGCTG
for RT-qPCR
validation
HrpB-F
GTCGATGAAGAAAGCCTCG
for RT-qPCR
validation
HrpB-R
CAGTCTTGCTCACCACTTG
for RT-qPCR
validation
HrpF-F
TAACCTCGATTCCACGCTCA
for RT-qPCR
validation
HrpF-R
CCTCAGCTGAGGTGCATCGATG
for RT-qPCR
validation
HrpL-F
GTGTTTCTCGAGGCGTTACG
for RT-qPCR
validation
HrpL-R
CGCGGATACATTGGGCGG
for RT-qPCR
validation
hrcC-F
CTTCAGCAGATGTCGATG
for RT-qPCR
validation
hrcC-R
CACAGGCTGATCGTTTCA
for RT-qPCR
validation
hrcN-F
GGCCGCTTATAAAACAGTG
for RT-qPCR
validation
| Primer Pair   | Sequence                  | Validation       |
|--------------|---------------------------|------------------|
| hrcN-R       | GGAACGCGTTTATAGCCTCG      | For RT-qPCR      |
| hrcR-F       | GCAGCCTCAAAGTCGTCATC      | validation       |
| hrcR-R       | CATGCGCTGGGTATTTTCCA      | For RT-qPCR      |
| avrB3-F      | TCTCCACACAGCAATACGT       | validation       |
| avrB3-R      | GGATCCTTTGTTCTGCGGC       | For RT-qPCR      |
| AvrRpm1-F    | TGCTGACACGAGTAATCCCA      | validation       |
| AvrRpm1-R    | TGATCTGTCATGAGTGCGGT      | For RT-qPCR      |
| Alg8-F       | GAGTTCTGTGAAGTGCGTG       | validation       |
| Alg8-R       | GCCATCGAGCAGCATGTTGAT     | For RT-qPCR      |
| Alg44-F      | CTGTACTTCGTACGACCCATGC    | validation       |
| Alg44-R      | CTTTGCCACGHTACCTTCACG     | For RT-qPCR      |
Swimming motility

Swarming motility

RNA-seq (fold-change)

Diameter in swimming plate (cm)

Diameter in swarming plate (cm)
