A YajQ-LysR-like, cyclic di-GMP-dependent system regulating biosynthesis of an antifungal antibiotic in a crop-protecting bacterium, *Lysobacter enzymogenes*

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**SUMMARY**

YajQ, a binding protein of the universal bacterial second messenger cyclic di-GMP (c-di-GMP), affects virulence in several bacterial pathogens, including *Xanthomonas campestris*. In this bacterium, YajQ interacts with the transcription factor LysR. Upon c-di-GMP binding, the whole c-di-GMP-YajQ-LysR complex is found to dissociate from DNA, resulting in virulence gene regulation. Here, we identify a YajQ-LysR-like system in the bacterial biocontrol agent *Lysobacter enzymogenes* OH11 that secretes an antifungal antibiotic, heat-stable antifungal factor (HSAF) against crop fungal pathogens. We show that the YajQ homologue, CdgL (c-di-GMP receptor interacting with LysR) affects expression of the HSAF biosynthesis operon by interacting with the transcription activator LysR. The CdgL-LysR interaction enhances the apparent affinity of LysR to the promoter region upstream of the HSAF biosynthesis operon, which increases operon expression. Unlike the homologues CdgL (YajQ)-LysR system in *X. campestris*, we show that c-di-GMP binding to CdgL seems to weaken CdgL-LysR interactions and promote the release of CdgL from the LysR-DNA complex, which leads to decreased expression. Together, this study takes the YajQ-LysR-like system from bacterial pathogens to a crop-protecting bacterium that is able to regulate antifungal HSAF biosynthesis via disassembly of the c-di-GMP receptor–transcription activator complex.

**Keywords:** antifungal antibiotic, CdgL, c-di-GMP, HSAF, Lysobacter, LysR.

**INTRODUCTION**

*Lysobacter enzymogenes* is an environmental predator of crop fungal pathogens (Christensen and Cook, 1978; Qian et al., 2009). As a crop-protecting bacterium, its primary weapon is the secreted antifungal antibiotic known as heat-stable antifungal factor (HSAF) via a new mode of action to target the sphingolipids biosynthesis pathway in the filamentous fungus *Aspergillus nidulans* (Li et al., 2006; Xu et al., 2015; Yu et al., 2007). Moreover, HSAF is synthesized by an operon containing more than ten genes in *L. enzymogenes*, wherein *lafB* (described initially as *hsaf* *pks/nrps*), the first gene of this operon, encodes a hybrid polyketide synthase/nonribosomal peptide synthetase that catalyses the linkage of one ornithine to two polyketide chains during HSAF assembly. This mechanism is unique among most known biosynthesis reactions of natural bacterial products (Chen et al., 2017; Lou et al., 2011; Wang et al., 2017).

Together, these unique features render HSAF an ideal candidate for the development of fungicides for crop protection.

We have shown previously that expression of the HSAF biosynthesis operon genes is controlled by multiple transcription factors, including three transcription activators (Clp, LysR and LarR) that regulate HSAF operon expression via direct binding to the operon promoter (Su et al., 2017, 2018; Wang et al., 2014) and two transcription repressors (LesR and LetR) (Qian et al., 2014; Wang et al., 2017). We also identified 4-hydroxybenzoic acid (4-HBA) as a diffusible chemical factor that promotes HSAF operon expression via binding to the transcription activator LysR (Su et al., 2017, 2018; Wang et al., 2014) and two transcription repressors (LesR and LetR) (Qian et al., 2014; Wang et al., 2017). We also identified 4-hydroxybenzoic acid (4-HBA) as a diffusible chemical factor that promotes HSAF operon expression via direct binding to the operon promoter (Su et al., 2017, 2018; Wang et al., 2014) and two transcription repressors (LesR and LetR) (Qian et al., 2014; Wang et al., 2017). Furthermore, cyclic di-GMP (c-di-GMP), a ubiquitous bacterial second messenger (Ross et al., 1987) that controls cellular processes through binding to intracellular receptor (or effectors) proteins and riboswitches, also controls HSAF biosynthesis according to our earlier studies, where we found that elevated levels of c-di-GMP inhibited antibiotic production at the level of HSAF biosynthesis operon transcription (Chen et al., 2017). We have identified a
signal transduction pathway involving the c-di-GMP-binding transcription activator, Clp, as the major target of c-di-GMP-dependent inhibition (Xu et al., 2018). Cyclic di-GMP decreases the affinity of Clp binding to the promoter region upstream of the HSAF biosynthesis operon, which lowers operon expression. Interestingly, Clp is sequestered by a membrane-bound c-di-GMP phosphodiesterase, PDE (LchP), and is released to function in response to an as yet unidentified environmental factor (Xu et al., 2018). In this study, we aimed to identify additional c-di-GMP receptor(s) controlling HSAP biosynthesis. To achieve this, we focused on YajQ, a newly identified c-di-GMP receptor protein regulating virulence in the plant pathogenic bacterium, Xanthomonas campestris. The YajQ homologues in human bacterial pathogens, Pseudomonas aeruginosa and Stenotrophomonas maltophilia, also function to control virulence. At the mechanistic level, YajQ was found to interact with a transcription factor, LysR, to regulate virulence gene expression in X. campestris (An et al., 2014). Notable, in this bacterium, c-di-GMP does not appear to inhibit YajQ-LysR interactions. Instead, it lowers the affinity of the YajQ-LysR complex to DNA, therefore the whole c-di-GMP-YajQ-LysR complex is believed to dissociate from DNA (An et al., 2014).

Herein, we identified a similar YajQ-LysR system through which elevated c-di-GMP concentration inhibits HSAP operon transcription. This system involves a c-di-GMP receptor protein, CdgL, the homologue of X. campestris YajQ, that interacts with a LysR-type transcription factor required for activating HSAP biosynthesis operon transcription. Our findings reveal that the mechanism of c-di-GMP-dependent regulation of the L. enzymogenes OH11 CdgL (YajQ)-LysR system appears to differ somewhat from the homologous system in X. campestris (An et al., 2014). In L. enzymogenes OH11, c-di-GMP most likely inhibits CdgL (YajQ)-LysR binary interactions and promotes the release of CdgL from the LysR-DNA complex to affect HSAP operon gene expression, while in X. campestris, as noted above, c-di-GMP seems to lower the affinity of the whole CdgL (YajQ)-LysR complex to DNA (An et al., 2014). Together, our findings expand the functionality and c-di-GMP-dependent mechanistic action of YajQ from regulating virulence in bacterial pathogens to antibiotic biosynthesis in a crop-protecting bacterium, L. enzymogenes OH11.

RESULTS

CdgL is a c-di-GMP-binding protein that affects gene expression in Lysobacter

The YajQ (Xc_3703) protein from X. campestris has recently been described as a c-di-GMP receptor that affects the virulence of this plant pathogen (An et al., 2014). Because Lysobacter is phylogenetically related to Xanthomonas (Christensen and Cook, 1978), we expected that it might also harbour a YajQ homologue. Indeed, according to BLASTP, the Le2538 protein encoded in the L. enzymogenes OH11 genome is 78% identical to the X. campestris YajQ. We have designated this protein CdgL (c-di-GMP receptor interacting with LysR) based on the findings of this study. We decided to investigate whether this putative new c-di-GMP receptor plays any role in HSAP biosynthesis or other aspects of L. enzymogenes OH11 physiology and metabolism.

First, we tested whether L. enzymogenes OH11 CdgL is a c-di-GMP receptor-like X. campestris YajQ. We constructed a glutathione S-transferase (GST)-fusion protein, GST-CdgL, which was soluble (Fig. S1). We measured c-di-GMP binding to GST-CdgL via microscale thermophoresis (MST) and found that GST-CdgL binds c-di-GMP with a moderate affinity ($K_d$ 14.62 µM) (Fig. 1A). It is noteworthy that c-di-GMP binding appears to be specific because no binding of GTP to GST-CdgL was observed (Fig. 1B).

We decided to pursue characterization of CdgL function via a genetic and transcriptomics approach. For this purpose, we generated an in-frame deletion in the cdgL gene via homologous recombination and analysed the effect of this mutation on the L. enzymogenes OH11 transcriptome. A comparative RNA-seq analysis uncovered 373 transcripts whose levels were affected (using a 1.5-fold expression change as a cut-off) by the lack of cdgl. Products of these genes were involved in a variety of cellular processes (Fig. S2 and Table S3). Interestingly, among the genes whose mRNA levels were decreased in the mutant, compared to the wild type, were several genes of the HSAP biosynthesis operon (Fig. 1C). Since we already knew that expression of this operon is sensitive to c-di-GMP concentrations (Xu et al., 2018) and CdgL binding to c-di-GMP (Fig. 1A), we decided to investigate the mechanism through which CdgL operates.

CdgL regulates HSAP biosynthesis at the transcription level

First, we verified the results of RNA-Seq by quantitative reverse transcription PCR (RT-qPCR). The mRNA transcript abundance of lafB, the first gene in the HSAP operon, in the ΔcdgL mutant was found to be one-fifth of that in the wild type. The lower mRNA level could be rescued by placing the plasmid-borne cdgl gene in the ΔcdgL mutant (Fig. 2A), which is consistent with the RNA-Seq results. To test whether CdgL-dependent changes in gene regulation affect the level of HSAP production, we quantified via high-performance liquid chromatography (HPLC) the amount of HSAP extracted from the wild-type and ΔcdgL cultures. The results shown in Fig. 2B reveal that HSAP levels produced by the mutant were several-fold lower than those produced by the wild type. The lower mRNA level could be rescued by placing the plasmid-borne cdgl gene in the ΔcdgL mutant (Fig. 2A), which is consistent with the RNA-Seq results.

To test whether CdgL-dependent changes in gene regulation affect the level of HSAP production, we quantified via high-performance liquid chromatography (HPLC) the amount of HSAP extracted from the wild-type and ΔcdgL cultures. The results shown in Fig. 2B reveal that HSAP levels produced by the mutant were several-fold lower than those produced by the wild type. The lower mRNA level could be rescued by placing the plasmid-borne cdgl gene in the Δcdgl mutant (Fig. 2A), which is consistent with the RNA-Seq results.

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Fig. 1  CdgL is a c-di-GMP receptor controlling expression of numerous genes in *Lysobacter enzymogenes*. (A) Characterization of c-di-GMP binding to CdgL via microscale thermophoresis (MST). GST-CdgL binds c-di-GMP with a $K_d = 14.62 \pm 2.04 \mu M$. FNorm is plotted on a linear y-axis in per mil (‰) against the total concentration of the titrated partner on a log$_{10}$ x-axis, as described previously (Seidel et al., 2013). (B) GST-CdgL does not bind GTP. (C) Expression changes in the HSAF biosynthetic genes in ΔcdgL compared to the wild type. The yellow arrow indicates genes encoding the hybrid polyketide synthase/nonribosomal peptide synthetase (NRPS/PKS, LafB) (Lou et al., 2011). Negative numbers indicate linear fold down-regulation of each gene in ΔcdgL compared to wild-type OH11.
the HSAF-production medium and found these curves to be virtually identical (Fig. S3). This result suggests that CdgL does not affect the growth but regulates HSAF synthesis in L. enzymogenes OH11.

To learn whether CdgL affects the HSAF operon transcription or mRNA stability, we constructed a lafB transcription fusion to the GUS reporter gene, gusA (Fig. 2C, plasmid pHSAF-GUS). The GUS activity in the ΔcdgL mutant grown in the HSAF-production medium was four-fold lower compared to the GUS activity in the wild type (Fig. 2C). These results suggest that CdgL is an important factor that controls HSAF biosynthesis by regulating transcription of the HSAF operon, possibly in a c-di-GMP-dependent manner.

CdgL interacts with the transcription activator of the HSAF biosynthesis operon, LysR, in vitro

Since CdgL protein lacks a distinct DNA-binding domain, it is unlikely to control transcription of HSAF operon directly. We thus hypothesized that CdgL affects HSAF operon transcription by acting upstream of one of the previously identified transcription regulators of this operon, Clp, MarR or LysR (Su et al., 2017, 2018; Xu et al., 2018). To test this hypothesis, we assessed the ability of CdgL to bind to each of these activators. Using a bacterial 2-hybrid (B2H) assay, we found that CdgL interacts with LysR (Fig. 3A) but not with Clp or MarR (Fig. S4). To validate the CdgL-LysR interactions, we carried out an additional MST test. The MST assay revealed that LysR-His6 bound to GST-CdgL with reasonably high affinity (Kd, 0.99 µM) (Fig. 3B). These results establish CdgL as a LysR-interacting partner.

The LysR protein is composed of two distinct domains, an N-terminal helix-turn-helix domain involved in DNA binding and a C-terminal domain involved in substrate (co-inducer) binding (Maddocks and Oyston, 2008). To identify the LysR domain primarily involved in interacting with CdgL, we made two truncated LysR derivatives and tested them using the B2H assay. One derivative comprises the substrate-binding domain of LysR and lacks the DNA-binding domain (Fig. 3C,D, LysR-LSD), while another derivative comprises the DNA-binding domain only (Fig. 3C,D, LysR-HD). CdgL interacted with the former but not the latter derivative (Fig. 3D), which suggests that CdgL binds to the LysR via the substrate-binding domain of LysR.

To verify that the in vitro observation of CdgL-LysR interaction is physiologically relevant, we used a genetic assay to test whether CdgL acts upstream of LysR. To this end, we knocked out the lysR gene in the ΔcdgL mutant background and compared the double ΔlysRΔcdgL mutant to the single lysR mutant in regard to lafB mRNA expression and HSAF production. According to these tests (Fig. 2A–C), the double and single mutants did not differ, which indicates that CdgL acts upstream of LysR.

CdgL enhances affinity of LysR to the HSAF operon promoter

To understand how CdgL binding to the LysR substrate-binding domain affects HSAF operon transcription, we considered two
possibilities. First, the CdgL-LysR interaction may increase the stability of the LysR protein, for example, by protecting LysR from proteolysis. This would increase the effective concentration of LysR and result in higher HSAF operon transcription. To test this possibility, we compared protein levels of the plasmid-encoded LysR-His6 in the wild type and \( \Delta \)cdgL mutant. We found these levels to be nearly identical (Fig. S5), which renders this hypothesis unlikely.

Second, the CdgL-LysR interaction may enhance LysR binding to the HSAF operon promoter, that is CdgL functions as a co-activator of LysR. We employed the electrophoretic mobility shift assay (EMSA) to test this possibility. As was expected from the CdgL protein architecture and our earlier work (Su et al., 2017), EMSA revealed that CdgL does not bind to the HSAF operon promoter (Fig. S5), while LysR does (Fig. 4A). Addition of the equimolar (2.5 µM) amount of CdgL did not seem to perturb the LysR-DNA complex (Fig. S7); however, addition of higher CdgL amounts (5–25 µM) resulted in an increased band intensity for the ternary CdgL-LysR-DNA complex, compared to the LysR-DNA complex (Fig. 4A). When higher amounts of CdgL were added, the mass of the CdgL-LysR-DNA complexes increased, suggesting that CdgL binds LysR in a non-stoichiometrical manner (Fig. 4A).

We quantified the binding affinity of LysR to the HSAF operon promoter in the presence of varying levels of CdgL. In the absence of CdgL, LysR bound to the promoter DNA with a high affinity (\( K_d, 25 \) nM) (Fig. 4B). The addition of equimolar amount of CdgL did little to perturb DNA binding (\( K_d, 29 \) nM) (Fig. 4B), which is consistent with the EMSA data (Fig. S7). However, in the presence of an excess of CdgL (25 µM), the apparent affinity of LysR to DNA increased by approximately nine-fold (\( K_d, 2.9 \) nM) (Fig. 4C), also in agreement with the EMSA data (Fig. 4A). We interpret the increase in apparent affinity as an indication that CdgL stabilizes the LysR-DNA complex.

Cyclic di-GMP binding to CdgL promotes disassembly of the CdgL-LysR-DNA complex

Since CdgL is capable of binding c-di-GMP (Fig. 1A), we tested whether c-di-GMP affected the CdgL-LysR interaction. First, we employed a B2H assay in two Escherichia coli host strains, XL-1 Blue, which has low c-di-GMP levels, and XL-1 Blue \( \Delta \) yhjH, which has high intracellular c-di-GMP levels due to the lack of the major c-di-GMP phosphodiesterase YhjH/PdeH (Fang et al., 2014). We observed no CdgL-LysR interactions in the high c-di-GMP strain, in contrast to the low c-di-GMP strain (Fig. 3A), which suggests that c-di-GMP inhibits CdgL-LysR interactions.

To validate the findings of the B2H system, we employed MST. We found that c-di-GMP indeed strongly inhibits CdgL-LysR
interactions. In the buffer containing 5 µM c-di-GMP, CdgL affinity to LysR was decreased by approximately 16-fold (Fig. 5A, Kd, 16.0 µM) compared to the buffer lacking c-di-GMP (Fig. 3C, 0.99 µM). In the 20-µM c-di-GMP buffer, CdgL-LysR interactions were not detectable (Fig. 5B). Importantly, 20 µM GTP had only a minor effect on the CdgL-LysR binding affinity (Fig. S8, Kd, 0.75 µM), therefore the inhibitory effect of c-di-GMP on CdgL-LysR interactions is specific. As an additional control, we tested whether LysR by itself binds c-di-GMP, but found no evidence of binding (Fig. S9). Together, these observations show that c-di-GMP binding to CdgL inhibits CdgL-LysR interactions.

We also tested how c-di-GMP binding affects the CdgL-LysR-DNA ternary complex. In an EMSA experiment, the CdgL-LysR-DNA ternary complex was stable in the presence of low (0.5–5 µM) c-di-GMP concentrations (Fig. S10). However, at higher (20 µM) concentrations, the amount of this complex decreased while the LysR-DNA binary complex reappeared (Fig. 5C). Notably, c-di-GMP did not interact with the test DNA probe (Fig. S6) or LysR (Fig. S9). Taken together, these data suggest that on binding to c-di-GMP, CdgL is released from the ternary complex, which effectively lowers the DNA binding affinity of LysR and decreases the extent of HSAF operon activation.

**DISCUSSION**

The bacterial second messenger c-di-GMP can bind to a plethora of receptor proteins, thus altering their enzymatic activities or affinity to other proteins and DNA (Chin et al., 2012; Fang et al., 2014; Jain et al., 2017; Xu et al., 2016; Yan et al., 2018). The latter mechanism, that is, c-di-GMP-dependent interactions with transcription factors, seems to be the major way through which c-di-GMP affects gene expression (Chin et al., 2010; Hickman and Harwood, 2008; Krasteva et al., 2010; Li and He, 2012; Li et al., 2018; Srivastava et al., 2003; Wilksch et al., 2011). Cyclic di-GMP binding to specific riboswitches is another mechanism of gene expression control (Smith et al., 2011). Rarely, the effect of c-di-GMP on gene expression does not involve direct c-di-GMP-transcription factor interactions. The best-understood example of the indirect effect involves a regulatory system controlling c-di-GMP-dependent activation of the genes encoding curli fimbriae and cellulose biosynthesis in *E. coli* and *Salmonella*. At low c-di-GMP levels, the c-di-GMP PDE (PdeR) sequesters the transcription factor MlrA. At high c-di-GMP levels, c-di-GMP binding triggers a conformational change in PdeR that leads to MlrA release and subsequent
Fig. 5  Effect of c-di-GMP on the CdgL-LysR and CdgL-LysR-DNA complexes. (A) Microscale thermophoresis (MST) showing that c-di-GMP (5 μM) lowers the affinity of CdgL to LysR to $K_a = 16.042 \pm 1.59 \mu M$ (from $K_a = 0.99 \mu M$). (B) MST showing that c-di-GMP (20 μM) completely abolishes the CdgL-LysR interactions. (C) Electrophoretic mobility shift assay (EMSA) showing that c-di-GMP (20 μM) disassembles the ternary CdgL-LysR-DNA complex resulting in the release of the binary LysR-DNA complex.
MlrA-activated expression of the downstream cascade, which ultimately results in the synthesis of curli fimbriae and cellulose. The PDE activity of PdeR contributes to lowering c-di-GMP levels and MlrA sequestration (Hengge et al., 2015).

The Lysobacter c-di-GMP-CdgL-LysR regulatory scheme deciphered in this study (Fig. 6) is similar to the scheme operating in enteric bacteria in that it also involves a c-di-GMP-dependent release of a transcription factor. However, there are notable differences between the two systems. In the absence of c-di-GMP, CdgL functions as a co-activator of the transcription activator LysR, while PdeR functions as an anti-activator of MlrA. CdgL enhances DNA binding by LysR and forms a tertiary CdgL-LysR-DNA complex, while PdeR is not involved in transcription regulation after the release of MlrA. Further, unlike PdeR, CdgL does not contribute to c-di-GMP degradation.

At the mechanistic level, our results suggest that CdgL increases, by several fold, the apparent affinity of LysR to the HSAF operon promoter (Fig. 4A,D). Cyclic di-GMP binding to CdgL changes its conformation, resulting in the disassembly of the CdgL-LysR-DNA complex and release of CdgL from the LysR-DNA complex (Fig. 5C). To our knowledge, such a mechanism has not been previously observed (Fig. 6). We do not yet fully understand whether CdgL promotes LysR dimerization, which seems to be necessary for DNA binding and stabilization of the LysR dimer-DNA complex, or works via a different mechanism. Obtaining additional details is complicated by the low solubility of CdgL and the need to employ a GST-CdgL fusion.

Intriguingly, although the L. enzymogenes OH11-CdgL-LysR system is similar to the homologous YajQ-LysR system in X. campesiris, their c-di-GMP-dependent regulation pattern on gene expression is not entirely the same. According to our results, in L. enzymogenes OH11, c-di-GMP binding to CdgL inhibits CdgL-LysR binary interactions (Figs 3A and 5B) and promotes the release of CdgL from the LysR-DNA complex (Fig. 5C). However, in X. campesiris, after c-di-GMP binding, the whole c-di-GMP-CdgL (YajQ)-LysR complex is believed to dissociate from DNA (An et al., 2014). Whether these differences reflect evolutionary divergence between the two bacteria or both mechanisms operate in each system, yet to a varying degree, remains to be elucidated.

Several concerns exist about the described regulatory system. One of them relates to the role of 4-HBA, which is one of the LysR ligands (Su et al., 2017). 4-HBA enhances HSAF operon expression and HSAF production (Su et al., 2017). The L. enzymogenes LysR, therefore, appears to have two co-activators, one of which is a small molecule, 4-HBA, and another a protein, CdgL, regulated by c-di-GMP. From the CdgL-LysR protein interaction interface analysis we surmised that the CdgL binds to the substrate-binding domain of LysR (Fig. 3C,D). We do not yet know whether 4-HBA and CdgL compete for binding to LysR or work synergistically as co-activators.

Another important issue concerns the existence of the second, Clp-mediated, pathway of c-di-GMP-dependent inhibition of HSAF operon expression. Clp is a ‘classical’ transcription activator whose DNA binding is inhibited by c-di-GMP (Xu et al., 2018), while c-di-GMP binds to CdgL to disrupt the CdgL-LysR complex formation (Fig. 5). These findings suggest that c-di-GMP binds to two different receptors at the same range, Clp and LysR, to control the same cellular process, HSAF biosynthesis, which has been termed ‘sustained sensing’ (Orr et al., 2016). What is the reason for the apparent redundancy for the c-di-GMP regulation? Is it justified by the fact that each system responds to a different source of c-di-GMP, which is regulated by certain specific stimulus? Do the Clp and CdgL-LysR regulatory systems compete or synergize in binding to the HSAF promoter region? Addressing these points is in progress in the laboratory.

Another important issue is CdgL itself. Is LysR the only partner of CdgL, mediating its effect on transcription, or does CdgL have multiple interaction partners? What other functions are under the CdgL control? The transcriptome data described at the beginning of this work (Fig. S2) may help answer some of these questions. However, based on the phenotypes affected by CdgL
in other bacteria, it appears that the realm of Cdgl (YajQ) targets is broad. In *X. campestris*, Cdgl (YajQ) affects motility gene expression (Su et al., 2017); in *P. aeruginosa*, it modulates temporal control of transcription in bacteriophage Φ6 (Qiao et al., 2008) and in all pathogens, such as *X. campestris*, *P. aeruginosa* and *S. maltophilia*, it controls virulence (Su et al., 2017). Our report adds c-di-GMP-dependent regulation of the antifungal antibiotic biosynthesis to this growing list of Cdgl targets.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains, plasmids and growth conditions**

The bacterial strains and plasmids used in this study are listed in Table S1. *Escherichia coli* was grown in LB medium with appropriate antibiotics at 37 °C. Unless stated otherwise, *L. enzymogenes* OH11 was grown in LB medium or 1/10 tryptic soy broth (TSB) at 28 °C with appropriate antibiotics: kanamycin (Km), 25 μg/mL, for mutant construction, and gentamicin (Gm), 150 μg/mL, for plasmid maintenance.

**Genetic methods**

Double-crossover homologous recombination was used to generate mutants in *L. enzymogenes* OH11, as described previously (Qian et al., 2012), using the primers listed in Table S2. In brief, two flanking regions of each gene were generated by PCR and cloned into the suicide vector pEX18Gm (Table S1). The final constructs were transformed into the wild-type strain by electroporation. The single-crossover recombinants were selected on LB plates supplemented with Km and Gm. The recombinants were cultured in LB medium without antibiotics for 6 h and subsequently plated on LB agar containing 10% (w/v) sucrose and Km. The sucrose-resistant, Km-resistant but Gm-sensitive colonies representing double crossovers were analysed further. In-frame gene deletions were verified by PCR using primers listed in Table S2.

Gene complementation constructs were generated as described earlier (Qian et al., 2014). In brief, a DNA fragment containing the full-length gene and its predicted promoter was amplified by PCR with different primer pairs (Table S2) and cloned into the broad-host-range vector pBRR1-MCS5 (Table S1). The plasmid was transformed into the wild-type or mutant strain by electroporation, and the transformants were selected on LB agar containing Km and Gm.

**RNA-Seq assay**

*Lyso bacter enzymogenes* OH11 and Δcdgl were grown in LB medium at 28 °C with 25 μg/mL Km. An overnight culture (2%) was inoculated into 1/10 TSB medium and grown at 28 °C until OD₆₀₀ = 1.0. The cells were then collected by centrifugation and treated with RNA Protect (Qiagen, Venlo, Netherlands), followed by lysozyme treatment. Total RNA was extracted using RNeasy Mini Kit (Qiagen). On-column DNase digestion with the RNase-free DNase Set (Qiagen) was used to remove DNA. The DNA contamination levels were assessed by using the Qubit dsDNA High Sensitivity (HS) (Thermo Fisher Scientific, Shanghai, China) assay (Picogreen dye) and the Qubit 2.0 Fluorometer (Invitrogen, Shanghai, China). The integrity of total RNA was assessed by using the Bioanlyser RNA analysis kit (Agilent Technologies, Santa Clara, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies). The Ribon- Zero Magnetic Bacterial Kit (Epcentric, Illumina, Beijing, China) was used to deplete 16S, 23S and 5S rRNAs from the samples. The first- and second-strand cDNA was synthesized by NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, Shanghai, China).

Three biological replicates of each strain were used. Libraries were produced using an Illumina TruSeq Stranded messenger RNA Sample Prep Kit. The libraries were sequenced using the Illumina HiSeq 2500 platform (Nanyang Technological University, Singapore) with a paired-end protocol and read lengths of 100 nt. The sequence reads were mapped onto the *L. enzymogenes* OH11 reference genome (GenBank accession number: RCTY00000000) (Lou et al., 2011) using the following criteria to filter out the unique sequence reads, with the maximum number of hits for a read of 1, minimum length fraction of 0.9, minimum similarity fraction of 0.8 and a maximum number of two mismatches. A constant of 1 was added to the raw transcript count value to avoid any problems caused by 0. The transcript count table was subjected to the DESeq package (Anders and Huber, 2010) of R/Bioconductor (Gentleman et al., 2004) for statistical analysis. The transcript counts were normalized to the sufficient library size. The differentially expressed genes were identified by performing a negative binomial test, and the transcripts were stringently determined as differentially expressed when they had a linear fold-change >1.5 and an adjusted P-value smaller than 0.05.

**Quantitative RT-PCR**

*Lyso bacter enzymogenes* OH11 were grown in 1/10 TSB and collected at OD₆₀₀ = 1.0. RNA was extracted using a Bacterial RNA Kit (OMEGA, Shanghai, China). Four hundred nanograms of RNA of each sample was then used to generate total cDNA using the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Shanghai, China), with the final cDNA diluted 20-fold with nuclease-free water. Primers are listed in Table S2. Quantitative RT-PCR was carried out by an Applied Biosystems 7500 system using 16S rRNA gene as an internal control, as described earlier (Qian et al., 2013, 2014). Each PCR (20 μL) contained 10 μL 2 × SYBR Premix Ex Taq (Takara), 0.4 μL of each primer, 0.4 μL Rox dye II (Takara), 2 μL of cDNA and 6.8 μL of nuclease-free water. The cycling protocol was: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s, before fluorescence detection followed by a melting curve determined with one cycle of 95 °C for 15 s, 60 °C for 60 s and 95 °C for 30 s. Data were analysed by Applied Biosystems 7500 software v. 2.0.6. Amplification specificity was assessed by
melting curve analysis. Relative fold change of the expression of individual genes was calculated using the 2−ΔΔCt method (Livak and Schmittgen, 2001). Data were analysed using SPSS v. 14.0 (SPSS Inc., Chicago, IL, USA). The hypothesis test of percentages (t-test, α = 0.05 or 0.01) was used to determine significant differences in gene expression.

**Promoter activity assay**

The 297-bp promoter region of the HSAF biosynthesis operon was PCR-amplified and fused with an 1824-bp, promoterless gusA whose product encodes a β-glucuronidase (GUS). The pHSAF-gusA fusion was cloned into the broad-host-range pBRR1-MCS5, in which the Pbac promoter is oriented opposite to the HSAF operon promoter. This construct was introduced by electroporation into the following strains: wild type, ΔcdgL, ΔlysR and ΔcdgLΔlysR. Then GUS activity was determined from the transformed strains, as described previously (Ferluga and Venturi, 2009). Data were analysed using SPSS v. 14.0. The hypothesis test of percentages (t-test, α = 0.05 or 0.01) was used to determine significant differences in GUS activity.

**Protein expression and purification**

We amplified the coding region of CdgL by PCR with the primers listed in Table S1 and cloned into vector pGEX-6p-1 to generate a GST-CdgL protein fusion. The resulting plasmid was transformed into E. coli BL21 (DE3) (Table S1) for protein expression and purification. The resultant strain was cultivated in LB medium (containing 100 µg/mL ampicillin) overnight at 37 °C. Three millilitres of the overnight culture was transferred into 200 mL fresh LB at 37 °C and grown with shaking at 200 rpm, until OD600 = 0.6. Isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma, St. Louis, MO, USA), was added to a final concentration, 0.5 mM. The culture was incubated for an additional 4 h at 37 °C. Cells were collected by centrifugation at 4 °C, resuspended in 25 mL phosphate-buffered saline (PBS) lysis buffer containing 10 mM protease inhibitor (PMSF, Sigma), and lysed by sonication (Sonifier 250; Branson Digital Sonifier, Danbury, USA). Following centrifugation at 15871 g at 4 °C for 30 min, the soluble protein was collected by incubation with GST beads for 30 min. The purified GST-CdgL protein was eluted with buffer containing glutathione. Protein purity was assessed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and protein concentration determined using a BCA assay kit (Sangon Biotech, Shanghai, China). Expression and purification of LysR-His in E. coli BL21 (DE3) was performed according to the protocol described earlier (Su et al., 2017).

**HSAF extraction and quantification**

HSAF was extracted from 20 mL L. enzymogenes OH11 cultures grown in 1/10 TSB for 24 h at 28 °C with shaking (at 200 rpm). The cells were removed by centrifugation (9391 g at 4 °C for 20 min). The culture supernatant was mixed with an equal volume of ethyl acetate and thoroughly shaken. The phases were separated, and the ethyl acetate phase was collected and evaporated to dryness. The HSAF-containing residue was dissolved in 200 µL of methanol and centrifuged (9391 g at 4 °C for 20 min), after which the supernatant was collected and applied to HPLC. Relative amounts of HSAF are expressed as peak intensity from the HPLC chromatogram per unit of OD600 according to earlier reports (Qian et al., 2013; Yu et al., 2007). Three biological replicates were used, and each was analysed in three technical replicates. Data were analysed using SPSS v. 14.0. The hypothesis test of percentages (t-test, α = 0.05 or 0.01) was used to determine significant differences in HSAF level.

**Bacterial two-hybrid assay**

BacterioMatch II Two-Hybrid system (BTH; Agilent Technologies) was used to determine potential interactions between two proteins. The approaches were described in our recent report (Xu et al., 2018). In brief, coding regions of target genes were cloned into pBT and pTRG plasmids and transformed into E. coli XL1-Blue MRF Kan or its ΔyjhH mutant. Plasmids pBT-GacS and pTRG-GacS (Table S1) were used as a positive control (Xu et al., 2018). The transformants containing empty pTRG and pBT vectors were used as a negative control. All co-transformants were spotted onto the selective medium and grown at 28 °C for 3–4 days. If there is a direct physical interaction between CdgL and its test partner(s), the transformed E. coli strain containing both vectors would be expected to grow well on the reference medium (selective medium, indicated as +3AT+Str2), which is the minimal medium (M9)-based medium supplemented with 5 mM 3AT, 2 µg/mL streptomycin, 12.5 µg/mL tetracycline, 34 µg/mL chloramphenicol and 30 µg/mL Km, as described previously (Xu et al., 2018). The LB agar is a nonselective medium (indicated as −3AT−Str2) containing 12.5 µg/mL tetracycline, 34 µg/mL chloramphenicol and 30 µg/mL Km, as reported in our recent work (Xu et al., 2018). The purpose of this medium is to ensure that both vectors are successfully transformed into the target E. coli strains.

**Electrophoretic mobility shift assay**

EMSA was performed as follows. Biotin-labelled probes of the 297-bp HSAF promoter region were synthesized by GENEWIZ (Suzhou, China). Probe and protein extract were incubated for 20 min at room temperature according to the specifications of the LightShift Chemiluminescent EMSA Kit (ThermoFisher). The binding mixture was loaded onto the polyacrylamide gel, electrophoresed, transferred to a nylon membrane and crosslinked as described in the manufacturer’s protocol. The biotinylated DNA fragments were detected by chemiluminescence using a VersaDoc imaging system (Bio-Rad, Philadelphia, USA).
Microscale thermophoresis assay

The protein–protein, protein–DNA, and c-di-GMP–protein affinities were determined by MST using Monolith NT.115 (NanoTemper Technologies, Germany) as described previously (Su et al., 2017; Xu et al., 2018). For the c-di-GMP-CdgL binding assay, the purified GST-CdgL was labelled with the fluorescent dye NT-647-NHS (NanoTemper Technologies) via amine conjugation. Constant concentration (500 μM) of the labelled target protein in a standard MST buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 0.05% Tween 20) was titrated against increasing concentrations of c-di-GMP, which were dissolved in diethylpyrocarbonate-treated water. The MST premium-coated capillaries (Monolith NT.115 MO-K005, Germany) were used to load the samples into the MST instrument at 25 °C using 80% MST power and 20% LED power. FNorm is plotted on a linear y-axis in per mil (%) against the total concentration of the titrated partner on a log₁₀ x-axis, as reported earlier (Seidel et al., 2013). For the LysR-DNA binding assay, a 297-bp HSAF operon promoter fragment was labelled with 5-carboxy-fluorescein (FAM). A constant concentration (10 μM) of the labelled probe in the MST buffer was titrated against increasing concentrations of LysR-His₈ dissolved in water. For the CdgL-LysR binding assay, the LysR-His₈ protein was labelled with the fluorescent dye NT-647-NHS via amine conjugation. A constant concentration (40 μM) of the labelled protein in the MST buffer was titrated against increasing concentrations of c-di-GMP, which were dissolved in diethylpyrocarbonate-treated water. The MST premium-coated capillaries (Monolith NT.115 MO-K005, Germany) were used to load the samples into the MST instrument at 25 °C using 80% MST power and 20% LED power. FNorm is plotted on a linear y-axis in per mil (%) against the total concentration of the titrated partner on a log₁₀ x-axis, as reported earlier (Seidel et al., 2013). For the LysR-DNA binding assay, a 297-bp HSAF operon promoter fragment was labelled with 5-carboxy-fluorescein (FAM). A constant concentration (10 μM) of the labelled probe in the MST buffer was titrated against increasing concentrations of LysR-His₈ dissolved in water. For the CdgL-LysR binding assay, the LysR-His₈ protein was labelled with the fluorescent dye NT-647-NHS via amine conjugation. A constant concentration (40 μM) of the labelled protein in the MST buffer was titrated against increasing concentrations of c-di-GMP, which were dissolved in diethylpyrocarbonate-treated water. The MST premium-coated capillaries (Monolith NT.115 MO-K005, Germany) were used to load the samples into the MST instrument at 25 °C using 80% MST power and 20% LED power. FNorm is plotted on a linear y-axis in per mil (%) against the total concentration of the titrated partner on a log₁₀ x-axis, as reported earlier (Seidel et al., 2013). For the LysR-DNA binding assay, a 297-bp HSAF operon promoter fragment was labelled with 5-carboxy-fluorescein (FAM). A constant concentration (10 μM) of the labelled probe in the MST buffer was titrated against increasing concentrations of LysR-His₈ dissolved in water. For the CdgL-LysR binding assay, the LysR-His₈ protein was labelled with the fluorescent dye NT-647-NHS via amine conjugation.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GenBank at https://www.ncbi.nlm.nih.gov/nuccore/, accession numbers MK012386 (L. enzymogenes OH11 YajQ; Le2538/Cdg, locus tag L1E_02538) and MG266894 (L. enzymogenes OH11 LysR family transcription factor gene, Le1703/LysR, locus tag = L1E_01703). The RNA-Seq raw data are available in NCBI Sequence Read Archive at https://www.ncbi.nlm.nih.gov/sra/, accession number SUB4688842.

REFERENCES

An, S., Qaly, D.L., McCarthy, Y., Murdoch, S.L., Ward, J., Febrer, M., Dow, J.M. and Ryan, R.P. (2014) Novel cyclic di-GMP effectors of the YajQ protein family control bacterial virulence. PLoS Pathog. 10, e1004429.

Anders, S. and Huber, W. (2010) Differential expression analysis for sequence count data. Genome Biol. 11, R106.

Chen, Y., Xia, J., Su, Z.H., Xu, G.G., Gomelsky, M., Qian, G.L. and Liu, F.Q. (2017) The regulator of type IV pili synthesis, PitR, from Lysobacter controls antifungal antibiotic production via a c-di-GMP pathway. Appl. Environ. Microbiol. 83, e03397–16.

Chen, K.H., Lee, Y.C., Tu, Z.L., Chen, C.H., Tseng, Y.H., Yang, J.M., Ryan, R.P., McCarthy, Y., Dow, J.M., Wang, A.H.J. and Chou, S.H. (2010) The cAMP receptor-like protein CLP is a novel c-di-GMP receptor linking cell-cell signaling to virulence gene expression in Xanthomonas campestris. J. Mol. Biol. 396, 646–662.

Chen, K.H., Kun, W.T., Wu, Y.J., Liao, Y.T., Yang, M.T. and Chou, S.H. (2012) Structural polymorphism of c-di-GMP bound to an EAL domain and in complex with a type II PIP domain protein. Acta Crystallogr. Sect. D: Biol. Crystallogr. 68, 1380–1392.

Christensen, P. and Cook, F.D. (1978) Lysobacter, a new genus of nonfruiting, gliding bacteria with a high base ratio. Int. J. Syst. Evol. Microbiol. 28, 367–393.

Fang, X., Ahmad, I., Blanka, A., Schottkowski, M., Cimdins, A., Galperin, M.Y., Romling, U. and Gomelsky, M. (2014) GLI, a new c-di-GMP-binding protein domain involved in regulation of cellulose synthesis in enterobacteria. Mol. Microbiol. 93, 439–452.

Ferluga, S. and Venturi, V. (2009) OryR is a LuxR-family protein involved in interkingdom signaling between pathogenic Xanthomonas oryzae pv. oryzae and rice. J. Bacteriol. 191, 890–897.

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y.C., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y. and Zhang, J.H. (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 5, R80.

Hengge, R., Galperin, M.Y., Ghigo, J.-M., Gomelsky, M., Green, J., Hughes, K.T., Jenal, U., Landini, P. and O’Toole, G.A. (2015) Systematic nomenclature for GGDEF and EAL domain-containing cyclic di-GMP turnover proteins of Escherichia coli. J. Bacteriol., 198, 7–11.

Hickman, J.W. and Harwood, C.S. (2008) Identification of FleQ from Pseudomonas aeruginosa as a c-di-GMP-responsive transcription factor. Mol. Microbiol. 69, 376–389.

Jain, R., Stiusarenko, O. and Kazmierczak, B.I. (2017) Interaction of the cyclic-di-GMP binding protein FimX and the type 4 pilus assembly ATPase promotes pilus assembly. PLoS Pathog. 13, e1006594.

Krasteva, P.V., Fong, J.C.N., Shikuma, N.J., Beyhan, S., Navarro, M.V., Yildiz, F.H. and Schottkowski, M., Cimdins, A., Galperin, M.Y., Romling, U. and Gomelsky, M. (2010) Systematic nomenclature for GGDEF and EAL domain-containing cyclic di-GMP turnover proteins of Escherichia coli. J. Bacteriol., 198, 7–11.

Li, S., Du, L., Yuen, G. and Harris, S.D. (2006) Distinct ceramide synthases regulate polarized growth in the filamentous fungus Aspergillus nidulans. Mol. Biol. Cell. 17, 1218–1227.
Li, W., Li, M., Hu, L., Zhu, J., Xie, Z., Chen, J.R. and He, Z.G. (2018) HpOR, a novel c-di-GMP effective transcription factor, links the second messenger’s regulatory function to the mycobacterial antioxidant defense. Nucleic Acids Res. 46, 3595–3611.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods, 25, 402–408.

Lou, L.L., Qian, G.L., Xie, Y.X., Hang, J.L., Chen, H.T., Zaleta-Rivera, K., Li, Y.Y., Shen, Y.M., Dussault, P.H., Liu, F.Q. and Du, L.C. (2011) Biosynthesis of HSAF, a tetrameric acid-containing macroalactam from Lysobacter enzymogenes. J. Am. Chem. Soc. 133, 643–645.

Maddock, S.E. and Oyston, P.C. (2008) Structure and function of the LysR-type transcriptional regulator (LTRR) family proteins. Microbiology, 154, 3609–3623.

Orr, M.W., Galperin, M.Y. and Lee, V.T. (2016) Sustained sensing as an emerging principle in second messenger signaling systems. Curr. Opin. Microbiol. 34, 119–126.

Qian, G.L., Hu, B.S., Jiang, Y.H. and Liu, F.Q. (2009) Identification and characterization of Lysobacter enzymogenes as a biological control agent against some fungal pathogens. Agric. Sci. China, 8, 68–75.

Qian, G.L., Wang, Y.S., Qian, D.Y., Fan, J.Q., Hu, B.S. and Liu, F.Q. (2012) Selection of available suicide vectors for gene mutagenesis using chaI (a chitinase encoding gene) as a new reporter and primary functional analysis of chaI in Lysobacter enzymogenes strain OH11. World J. Microbiol. Biotechnol. 28, 549–557.

Qian, G.L., Wang, Y.L., Liu, Y.R., Xu, F.F., He, Y.W., Du, L.C., Venturi, V., Fang, J.Q., Hu, B.S. and Liu, F.Q. (2013) Lysobacter enzymogenes uses two distinct cell-cell signaling systems for differential regulation of secondary-metabolite biosynthesis and colony morphology. Appl. Environ. Microbiol. 79, 6604–6616.

Qian, G.L., Xu, F.F., Venturi, V., Du, L.C. and Liu, F.Q. (2014) Roles of a solo LuxR in the biological control agent Lysobacter enzymogenes strain OH11. Phytopathology, 104, 224–231.

Qiao, X., Sun, Y., Qiao, J. and Mindich, L. (2008) The role of host protein YajQ in the temporal control of transcription in bacteriophage Φ6. Proc. Natl. Acad. Sci. USA, 105, 15956–15960.

Ross, P., Weinhouse, H., Aloni, Y., Michaeli, D., Weinberger-Ohana, P., Mayer, R., Braun, S., Vroom, E.D., Marel, G.A., Boom, J.H. and Benzmian, M. (1987) Regulation of cellulose synthesis in Acetobacter xylinum by cyclic di-guanylic acid. Nature, 325, 279–281.

Seidel, S.A., Dijkman, P.M., Lea, W.A., Van den Bogaart, G., Jerabek-Willemsen, M., Lazic, A., Joseph, J.S., Srinivasan, P., Baaske, P., Simeonov, A., Katritch, I., Melo, F.A., Ladbury, J.E., Schreiber, G., Watts, A., Braun, D. and Duhr, S. (2013) Microscale thermophoresis quantifies biomolecular interactions under previously challenging conditions. Methods, 59, 301–315.

Smith, K.D., Shanahan, C.A., Moore, E.L., Simon, A.C. and Strobel, S.A. (2011) Structural representation of differential ligand recognition by two classes of bis-(3′-5′)-cyclic dimeric guanosine monophosphate-binding riboswitches. Proc. Natl. Acad. Sci. USA, 108, 7757–7762.

Srivastava, D., Hsieh, M.L., Khataokar, A., Neiditch, M.B. and Waters, C.M. (2011) Cyclic di-GMP inhibits Vibrio cholerae motility by repressing induction of transcripton and inducing extracellular polysaccharide production. Mol. Microbiol. 90, 1262–1276.

Su, Z.H., Chen, H.F., Wang, P., Tombosa, S., Du, L.C., Han, Y., Shen, Y.M., Qian, G.L. and Liu, F.Q. (2017) 4-Hydroxynicosa-12(Z)-enol is a diffusible factor that connects metabolic shikimate pathway to the biosynthesis of a unique antifungal metabolite in Lysobacter enzymogenes. Mol. Microbiol. 104, 163–178.

Su, Z.H., Han, S., Xu, Z.Q., Qian, G.L. and Liu, F.Q. (2018) Heat-stable antifungal factor (HSAF) biosynthesis in Lysobacter enzymogenes is controlled by the interplay of two transcription factors and a diffusible molecule. Appl. Environ. Microbiol. 84, e01754–17.

Wang, P., Chen, H.F., Qian, G.L. and Liu, F.Q. (2017) LetR is a TetR family transcription factor from Lysobacter controlling antifungal antibiotic biosynthesis. Appl. Microbiol. Biotechnol. 101, 3273–3282.

Wang, Y., Zhao, Y., Zhang, J., Zhao, Y., Shen, Y., Su, Z., Xu, G., Du, L., Huffman, J.M., Venturi, V., Qian, G. and Liu, F. (2014) Transcriptomic analysis reveals new regulatory roles of Cip signaling in secondary metabolite biosynthesis and surface motility in Lysobacter enzymogenes OH11. Appl. Microbiol. Biotechnol., 98, 9009–9020.

Wilksch, J.J., Yang, J., Clements, A., Gabbe, J.L., Short, K.R., Cao, H., Cavaliere, R., James, C.E., Whitchurch, C.B., Schembri, M.A., Chua, M.L.C., Liang, Z.X., Wijbrug, O.L., Jenney, A.W., Lithgow, T. and Strugnell, R.A. (2011) MrkH, a novel c-di-GMP-dependent transcriptional activator, controls Klebsiella pneumoniae biofilm formation by regulating type 3 fimbriae expression. PLoS Pathog. 7, e1002204.

Xu, L.X., Wu, P., Wright, S.J., Du, L.C. and Wei, X.Y. (2015) Bioactive polycyclic tetrameric macroalactams from Lysobacter enzymogenes and their absolute configurations by theoretical ECD calculations. J. Nat. Prod. 78, 1841–1847.

Xu, L., Xin, L., Zeng, Y., Yam, J.K., Ding, Y., Venkataramani, P., Cheang, Q.W., Yang, X.B., Tang, X.H., Zhang, L.H., Chiam, K.H., Yang, L. and Liang, Z.X. (2016) A cyclic di-GMP-binding adaptor protein interacts with a chemotaxis methyltransferase to control flagellar motor switching. Sci. Signaling, 9, ra102.

Xu, G.G., Han, S., Hsu, C.M., Chiu, K.H., Chou, S.H., Gomelsky, M., Qian, G.L. and Liu, F.Q. (2018) Signaling specificity in the c-di-GMP-dependent network regulating antibiotic synthesis in Lysobacter. Nucleic Acids Res. 46, 9276–9288.

Yan, X.F., Xin, L.Y., Yen, J.T., Zeng, Y.K., Jin, S.Y., Cheang, Q.W., Fong, R.A.C.Y., Chiam, K.H., Liang, Z.X. and Gao, Y.G. (2018) Structural analyses unravel the molecular mechanism of cyclic di-GMP regulation of bacterial chemotaxis via a PilZ adaptor protein. J. Biol. Chem. 293, 100–111.

Yu, F.G., Zaleta-Rivera, K., Zhu, X.C., Huffman, J., Millet, J.C., Harris, S.D., Yuan, G., Li, X.C. and Du, L.C. (2007) Structure and biosynthesis of heat-stable antifungal factor (HSAF), a broad-spectrum antimycotic with a novel mode of action. Antimicrob. Agents Chemother. 51, 64–72.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Fig. S1 Purified GST-tagged CdgL.

Fig. S2 Gene ontology categories of differentially expressed genes (373 in total) when cdg1 was knocked out in Lysobacter enzymogenes OH11.

Fig. S3 The Δcdg1 mutation does not perturb growth of Lysobacter enzymogenes in the HSAF production medium.

Fig. S4 An Escherichia coli-based bacterial 2-hybrid assay showing the lack of CdgL interactions with Cip or MarR.

Fig. S5 The Δcdg1 mutation does not affect LysR protein abundance in Lysobacter enzymogenes.

Fig. S6 CdgL and c-di-GMP do not bind to the HSAF operon promoter.

Fig. S7 Effect of low amount of CdgL on the LysR-DNA complex.

Fig. S8 GTP does not affect the CdgL-LysR protein complex.

Fig. S9 Cyclic di-GMP does not bind to LysR-His6.

Fig. S10 Effect of c-di-GMP on the CdgL-LysR-DNA complex.

Table S1 Strains and plasmids used in this study

Table S2 Primers used in this study

Table S3 Summary of RNA-Seq data between wild-type Lysobacter enzymogenes OH11 and the cdg1 deletion mutant