Coordinated control of the type IV pili and c-di-GMP-dependent antifungal antibiotic production in *Lysobacter* by the response regulator PilR

Kangwen Xu1 | Danyu Shen1 | Nianda Yang1 | Shan-Ho Chou2 | Mark Gomelsky3 | Guoliang Qian1

**Abstract**

In the soil gammaproteobacterium *Lysobacter enzymogenes*, a natural fungal predator, the response regulator PilR controls type IV pili (T4P)-mediated twitching motility as well as synthesis of the heat-stable antifungal factor (HSAF). Earlier we showed that PilR acts via the second messenger, c-di-GMP; however, the mechanism remained unknown. Here, we describe how PilR, c-di-GMP signalling, and HSAF synthesis are connected. We screened genes for putative diguanylate cyclases (c-di-GMP synthases) and found that PilR binds to the promoter region of *lchD* and down-regulates its transcription. The DNA-binding affinity of PilR, and therefore its repressor function, are enhanced by phosphorylation by its cognate histidine kinase, PilS. The *lchD* gene product is a diguanylate cyclase, and the decrease in LchD levels shifts the ratio of c-di-GMP-bound and c-di-GMP-free transcription factor Clp, a key activator of the HSAF biosynthesis operon expression. Furthermore, Clp directly interacts with LchD and enhances its diguanylate cyclase activity. Therefore, the PilS–PilR two-component system activates T4P-motility while simultaneously decreasing c-di-GMP levels and promoting HSAF production via the highly specific LchD–c-di-GMP–Clp pathway. Coordinated increase in motility and secretion of the "long-distance" antifungal weapon HSAF is expected to ensure safer grazing of *L. enzymogenes* on soil or plant surfaces, unimpeded by fungal competitors, or to facilitate bacterial preying on killed fungal cells. This study uncovered the mechanism of coregulated pili-based motility and production of an antifungal antibiotic in *L. enzymogenes*, showcased the expanded range of functions of the PilS–PilR system, and highlighted exquisite specificity in c-di-GMP-mediated circuits.

**Keywords**

antibiotic, biocontrol, c-di-GMP, *Lysobacter*, PilS-PilR
1 INTRODUCTION

Species of the gammaproteobacterial genus Lysobacter are common inhabitants of agricultural soils, water, and plant surfaces. They are known for their ability to prey on other microorganisms by means of secreted antibiotics and lytic enzymes (Christensen & Cook, 1978; Puopolo et al., 2018). L. enzymogenes is the best-studied species of the genus and a proficient predator of filamentous fungi and oomycetes (Li et al., 2006; Qian et al., 2009). The main antifungal agent secreted by L. enzymogenes strain OH11 is the antibiotic known as heat-stable antifungal factor, HSAF (Li et al., 2006; Qian et al., 2013; Yu et al., 2007). The HSAF biosynthesis is controlled primarily at the level of transcription of the HSAF biosynthesis operon, and several transcription factors affecting transcription have been identified (Chen et al., 2017; Su et al., 2017; Wang et al., 2014). One of these factors is PilR, a response regulator of the PilS-PilR two-component system typically associated with type IV pili (T4P) synthesis (Chen et al., 2017; Hobbs et al., 1993; Kilmury & Burrows, 2016). PilR binds to the promoter region of the pilA gene encoding the T4P major subunit (Hernández-Eligio et al., 2017; Jin et al., 1994). Phosphorylation by its cognate histidine kinase, PilS, results in higher DNA-binding affinity, activation of pilA expression, enhanced T4P assembly, and induction of twitching motility (Kilmury & Burrows, 2016, 2018). This mode of regulation is conserved in T4P-producing bacteria, and L. enzymogenes is no exception to this rule (Boyd et al., 1994; Chen et al., 2017; Hernández-Eligio et al., 2017; Kehl-Fie et al., 2009; Wu & Kaiser, 1997). Our earlier studies revealed that PilR affects HSAF production by decreasing cellular concentration of c-di-GMP via an unknown mechanism (Chen et al., 2017; Xu et al., 2018).

Cyclic di-GMP is a ubiquitous bacterial second messenger (Ross et al., 1987) that controls numerous cellular processes via protein effectors (or receptors) and riboswitches (Jain et al., 2017; Römling et al., 2013). Cyclic di-GMP is synthesized by diguanylate cyclases (DGCs) containing the GGDEF catalytic domain via the condensation of two GTP molecules (Paul et al., 2004; Ryjenkov et al., 2005). It is broken down by c-di-GMP-specific phosphodiesterases (PDEs) that contain EAL (Christen et al., 2005; Schmidt et al., 2005; Tamayo et al., 2005) or HD-GYP domain proteins presumably involved in c-di-GMP synthesis or degradation (Ren et al., 2020). Three of these proteins, RpfG, LchP (both PDEs), and WspR (a DGC), regulate HSAF biosynthesis (Han et al., 2015; Xu et al., 2018, 2021) by means of two c-di-GMP-binding protein effectors, Clp and CdgL (Han et al., 2020; Xu et al., 2018). Clp is a transcription activator of the operon. In the c-di-GMP-free state, Clp binds to two sites, designated PA and PB, in the HSAF biosynthesis operon promoter region (Xu et al., 2018). Cyclic di-GMP binding decreases Clp affinity to DNA, which results in lower HSAF operon expression (Xu et al., 2018). Clp specifically interacts with LchP, and such interaction stimulates the PDE activity of LchP (Xu et al., 2018). CdgL (known as YajQ in Xanthomonas campestris) does not bind DNA, in contrast to Clp (Han et al., 2020). It functions as a coactivator, by binding to LysR, the transcription activator of the HSAF biosynthesis operon (Han et al., 2020). The c-di-GMP-bound CdgL promotes disassociation of the CdgL-LysR complex, which leads to lower HSAF operon expression. CdgL interacts with WspR, which is dedicated to control CdgL but not Clp loading with c-di-GMP (Xu et al., 2021).

We know that L. enzymogenes PilR promotes HSAF operon expression by down-regulating intracellular c-di-GMP concentration but how this is accomplished remained puzzling. In this study, we show that PilR acts primarily through LchD (Lysobacter c-di-GMP and HSAF-associated DGC), a previously uncharacterized DGC. PilR binds to the lchD promoter and decreases lchD expression, thus leading to lower LchD abundance. We further show that LchD specifically interacts with the transcription activator Clp, but not with CdgL. Lower LchD levels shift the ratio of c-di-GMP-loaded and c-di-GMP-free Clp toward the latter, which stimulates HSAF biosynthesis operon expression and HSAF secretion. Our study, therefore, reveals the mechanism through which the PilS-PilR two-component regulatory system coactivates T4P-dependent motility and HSAF production via the LchD-c-di-GMP-Clp cascade. The uncovered co-regulation may improve predation efficiency by L. enzymogenes grazing on the soil and plant surfaces infected with fungi and oomycetes.

2 RESULTS

2.1 PilR binds to the promoter of the lchD gene encoding a DGC

Because PilR was shown earlier to decrease c-di-GMP concentration, we hypothesized that it either represses expression of a DGC gene or activates expression of a PDE gene. To test this hypothesis, we investigated PilR binding to promoter regions of 10 putative DGC genes as well as two PDE genes (lchP and rpfG) previously shown by us to affect c-di-GMP concentration under the experimental conditions used (Figure 1a) (Ren et al., 2020; Xu et al., 2018). The screen involving a bacterial one-hybrid (B1H) system revealed that PilR binds to a single region (P\textsubscript{LchD}) upstream of the le3756 gene, which we named lchD (Figure 1b). To validate this observation, we overexpressed PilR and purified it as a C-terminal His-tag fusion from Escherichia coli. Microscale thermophoresis (MST) experiments (Seidel et al., 2013) revealed that the PilR-His fusion binds to the 300-bp DNA fragment, P\textsubscript{LchD}, with $K_D$ 0.56 μM (Figure 1c), but it does not bind to promoter regions upstream of the remaining 11 DGC and PDE genes (Figure 1d).

To localize the putative PilR binding site, we searched the P\textsubscript{LchD} sequence for the consensus binding site of Pseudomonas aeruginosa PilR, 5’-TGT-(N)\textsubscript{1-2}-ACA, but did not identify a close match (Figure S1). This suggests that the L. enzymogenes PilR binding site may have diverged from that of its P. aeruginosa homolog, or that the site in P\textsubscript{LchD} is highly divergent. To narrow down the binding region, we split P\textsubscript{LchD} in half, creating two DNA probes, P\textsubscript{LchD-1} and P\textsubscript{LchD-2} (Figure 2a). PilR bound only to the P\textsubscript{LchD-2} probe (150 bp DNA) in both B1H (Figure 2b) and MST assays (Figure 2c). The P\textsubscript{LchD-2} fragment was...
further split into three smaller, 50-bp probes, PLchD-2a, PLchD-2b, and PLchD-2c (Figure 2a), each of which was tested for PilR-His binding. As shown in Figure 2d, PilR-His bound to PLchD-2a, but not to PLchD-2b or PLchD-2c determined by electrophoretic mobility shift assay (EMSA). The PilR-His – PLchD-2a complex could be competitively inhibited to some extent by the unlabelled probe provided.
in excess (Figure 2d), which further indicates that the interaction is specific. According to the MST assay, PilR-His binds to P\textsubscript{LchD}\textsubscript{-2}, but not P\textsubscript{LchD}\textsubscript{-1} as detected by bacterial one-hybrid system. CK\textsuperscript{+} and CK\textsuperscript{−} are same as in Figure 1b. (c) Characterization of the PilR binding affinity (K\textsubscript{d} 0.56 μM) to P\textsubscript{LchD}\textsubscript{-2} via microscale thermophoresis (MST). No binding of PilR to P\textsubscript{LchD}\textsubscript{-1} was observed. (d) Electrophoretic mobility shift assay showing PilR-His binding to P\textsubscript{LchD}\textsubscript{-2a}, but not P\textsubscript{LchD}\textsubscript{-2b} or P\textsubscript{LchD}\textsubscript{-2c}. (e) PilR binding (K\textsubscript{d} 0.54 μM) to P\textsubscript{LchD}\textsubscript{-2a}, but not P\textsubscript{LchD}\textsubscript{-2b} or P\textsubscript{LchD}\textsubscript{-2c}, as detected by MST. (f) DGC activity as motility inhibition in semisolid agar. Escherichia coli MG1655 expressing lchD and the lchD\textsuperscript{GGAAV} mutant from the arabinose-inducible promoter (plasmid pBAD; Table S1). Slr1143, a potent DGC used as positive control. Average data ± SD from three experiments. *p < .01
2.2 | Phosphorylation enhances DNA-binding affinity of PilR resulting in stronger lchD expression down-regulation and higher HSAF production

The REC domain of the L. enzymogenes PilR carries a conserved aspartate residue, D55 (Figure S3), presumed to be phosphorylated by the histidine kinase PilS (Hernández-Elio et al., 2017; Klumury & Burrows, 2016). To investigate the role of phosphorylation in DNA binding, we generated two PilR variants. One, PilRD55A, was expected to be inactive, while the other one, PilRDSSE, was expected to be constitutively active, mimicking the phosphorylated state. The mutant PilR proteins, which were purified as C-terminal His-tag fusions, similar to the wild-type PilR, were assayed for binding to PrLchD-2a by means of MST. The binding affinity of the inactive PilRD55A mutant (Kₐ 0.56 µM) was similar to that of the wild-type PilR, whereas the constitutively active PilRDSSE mutant showed an approximately threefold higher affinity (Kₐ 0.14 µM). DNA-binding affinity of the wild-type PilR was increased by approximately threefold (Kₐ 0.15 µM) after treatment with acetyl phosphate, a phosphorylation agent specifically targeting aspartate residues in the REC domains of response regulators (Hickman et al., 2005; McCleary & Stock, 1994) (Figure 3a). Similar results were observed when DNA binding by the PilR derivatives was monitored by means of EMSA (Figure 3b). These tests demonstrate that phosphorylation seems to significantly increase DNA binding by PilR to the upstream region of the lchD gene.

To verify the physiological significance of the biochemical analyses described above, we carried out genetic and phenotypic assays. First, we observed that deletion of the pilR gene, ΔpilR, resulted in an approximately threefold increase in lchD mRNA levels, consistent with the expectation that PilR binding down-regulates lchD expression. This defect could be complemented by the chromosomal insertion of the native pilR gene (OH11-pilR) or the insertion of the constitutively active pilRDSSE allele (OH11-pilRDSSE), whereas complementation with the chromosomally inserted pilRDS5A allele (OH11-pilRDS5A) was less effective (Figure 3c). The western blot analysis indicated that the PilR protein abundance was not significantly affected by either of the two mutations, D55A or D55E (Figure S4). Therefore, PilR acts as repressor of lchD gene expression, whose function is most probably enhanced by phosphorylation. Next, we assayed the effect of PilR phosphorylation on HSAF biosynthesis operon expression by measuring mRNA abundance of lafB, the first gene in the operon. LafB mRNA levels, measured by quantitative reverse transcription PCR (RT-qPCR), were decreased in the ΔpilR mutant, compared to the wild type. The wild-type pilR or pilRDSSE alleles restored LafB mRNA levels whereas the pilRDS5A allele did not (Figure 3d), in line with the mutation effects on lchD expression.

PilR is expected to be phosphorylated by PilS. To test this assumption, we blocked PilS autophosphorylation by substituting the conserved histidine with alanine, H328A (Figure S5). The chromosomal pilSH328A mutant (OH11-pilS(H328A)) had significantly higher lchD transcript abundance (Figure S6), thus supporting the conclusion that PilR phosphorylation seems to enhance lchD repression.

To test how PilR-mediated lchD gene repression affects LchD protein levels, we ran a western blot. We found that LchD-FLAG abundance was increased by approximately twofold in the ΔpilR and OH11-pilRDS5A strains compared to the strains containing the native pilR gene, OH11, and OH11-pilR. As expected, LchD-FLAG abundance in strain OH11-pilRDS5E was lower than in OH11 and OH11-pilR (Figure 3e). These results show that PilR appears to decrease LchD abundance in a phosphorylation-dependent manner.

Lastly, we investigated how PilR-mediated down-regulation of LchD abundance affects HSAF production. HSAF levels were measured either by high performance liquid chromatography (HPLC) or by its antifungal activity. We found HSAF amounts in the ΔpilR mutant to be <20% of the amounts produced by OH11. Complementation of the mutant with the wild-type pilR (OH11-pilR) or pilRDSSE (OH11-pilRDSSE) alleles restored HSAF production, whereas complementation with pilRDS5A (OH11-pilRDS5A) was ineffective (Figure 3f). HSAF levels were also lower in the ΔpilS mutant and strain OH11-pilSH328A (Figure S7). The HPLC-based HSAF measurements were consistent with the outcomes of the antifungal plate assay. HSAF-containing growth medium from the OH11-pilR and OH11-pilRDS5E strains effectively inhibited growth of the filamentous fungal pathogen Valsa pyri, whereas medium from the ΔpilR, OH11-pilRDS5A, and OH11-pilSH328A strains did not (Figures 3g,h and 5B). Taken together, these data establish that PilR phosphorylation plays key roles in stimulating HSAF production by decreasing lchD expression and lowering LchD abundance.

2.3 | Importance of the PilR-LchD pathway for HSAF production

Here, we wanted to evaluate the relative contribution of the PilR-LchD pathway on HSAF production. To this end, we measured HSAF amounts in the ΔpilR ΔlchD double mutant. The double mutant produced c.70% HSAF compared to the wild type. Importantly, when we mutated randomly chosen DGC genes in the ΔpilR background, HSAF production remained essentially unchanged (Figure 4a). Similarly, lafB mRNA abundance was mostly rescued in the ΔpilR ΔlchD double mutant but not in the ΔpilR mutant containing deletions in other DGC genes (Figure 4b). The same was true for the HSAF levels (Figure 4c,d). These results indicate that the effect of PilR on HSAF production is mediated primarily (c.70%) through LchD, but additional targets may exist.

To support the notion that the effect of PilR is mediated via changes in c-di-GMP concentration, we expressed LchD as well as its inactive variant LchDGGAAV from a plasmid in the ΔpilR background. Overexpression of LchD, but not LchDGGAAV, significantly decreased HSAF production, similar to the decrease caused by the potent DGC, Srl1143. The plasmid-expressed wild-type LchD and the LchDGGAAV variant were present at similar levels (Figure S9). The replacement of the chromosomal lchD with the lchDGGAAV variant in the ΔpilR background resulted in drastically
higher HSAF production compared to the ΔpilR mutant (Figure 4h). Furthermore, the ΔlchD deletion in the ΔpilS background increased HSAF production, similar to the phenotype of the ΔpilR ΔlchD mutant (Figure S10). These results confirm that PilR up-regulates HSAF production by decreasing c-di-GMP synthesis, primarily through LchD.

2.4 | LchD interacts with the c-di-GMP-binding transcription activator Clp

To explain how a moderate decrease in LchD protein abundance (Figure 3e) can evoke large changes in HSAF operon expression and HSAF production (Figure 3d,f), we hypothesized that LchD directly
“loads” with c-di-GMP one of the factors controlling HSAF operon expression. This hypothesis is consistent with several recent studies showing direct interactions of c-di-GMP effectors with specific DGCs and PDEs (Dahlstrom et al., 2015; Giacalone et al., 2018; Hobley et al., 2012; Xu et al., 2018; Yang et al., 2017).

First, we investigated whether PilR is such a factor. Some members of the AAA protein superfamily to which PilR belongs are known to bind c-di-GMP, for example P. aeruginosa FleQ (Baraquet & Harwood, 2016; Baraquet et al., 2012; Hickman & Harwood, 2008). We performed MST experiments using unphosphorylated or phosphorylated forms of PilR-His, but we did not know how PilR, c-di-GMP, and HSAF are connected.

To uncover the mechanism, we tested the hypothesis that PilR regulates expression of one or more DGC or PDE genes. After screening promoter fusions to the relevant DGC and PDE genes, we found that PilR binds upstream of a single gene, lchD presumably encoding a DGC (Figure 1). We showed that PilR binding results in down-regulation of lchD expression (Figure 2a–e) and that LchD has DGC activity, we expressed the LchDGGAAV (plasmid pBAD; Table S1), but not in the absence of arabinose. To verify this hypothesis, we performed the B2H assay in two heterologous hosts, E. coli. To accomplish this, we performed the B2H assay in two E. coli strains, one of which (XL1-Blue) has low and another (XL1-Blue ΔpilR) high intracellular c-di-GMP concentrations. Cyclic di-GMP concentration in the ΔpilR strain is high because it lacks YhjH/PdeH, a dominant PDE (El Mouali et al., 2017; Fang et al., 2014; Hengge et al., 2016). The B2H assay detected LchD–Clp interactions only in the low c-di-GMP strain (Figure 5g), suggesting that high levels of c-di-GMP inhibit LchD–Clp interactions. This conclusion was confirmed by MST, where binding affinity of the cytoplasmic LchD-His to Clp-GST was found to be approximately fourfold lower in the presence of 2 µM c-di-GMP compared to no c-di-GMP. An analog of c-di-GMP, c-di-AMP, at 2 µM concentration, had negligible effect on the LchD–Clp interaction (Figure 5h), verifying the specificity of the effect of c-di-GMP. It is worth noting that the 2 µM c-di-GMP concentration used in the MST experiment is within the presumed physiological range, because the K_d of Clp–c-di-GMP binding is 1.5 µM (Xu et al., 2018). These results suggest loading of Clp with c-di-GMP probably results in dissociation of the LchD–Clp complex.

3 | DISCUSSION

In this work, we pursued the unexpected finding that PilR, a transcription regulator of T4P synthesis and twitching motility in L. enzynogenes, also activates production of an antifungal antibiotic HSAF (Chen et al., 2017; Lin et al., 2020). We showed previously that the effect of PilR is mediated by c-di-GMP signalling (Chen et al., 2017), but we did not know how PilR, c-di-GMP, and HSAF are connected. To uncover the mechanism, we tested the hypothesis that PilR regulates expression of one or more DGC or PDE genes. After screening promoter fusions to the relevant DGC and PDE genes, we found that PilR binds upstream of a single gene, lchD presumably encoding a DGC (Figure 1). We showed that PilR binding results in down-regulation of lchD expression (Figure 2a–e) and that LchD has DGC activity, we expressed the LchDGGAAV (plasmid pBAD; Table S1), but as measured by microscale thermophoresis. PilR-His, PilR containing the D55E mutation mimicking the phosphorylation state (K_d 0.15 µM); PilRGGAAV-His, PilR-His containing the D55A mutation disabling phosphorylation (K_d 0.46 µM). (b) PilR phosphorylation stimulates binding to P_lchD-2a, as measured by electrophoretic mobility shift assay. (c) PilR phosphorylation enhances lchD repression, as measured by quantitative reverse transcription PCR (RT-qPCR). OH11, wild type; ΔpilR, OH11, in-frame pilR deletion; strains OH11- ΔpilR, OH11- ΔpilRGGAAV and OH11- ΔpilRGGAAV contain chromosomal insertions of the native pilR gene, pilRGGAAV and pilRGGAAV alleles, respectively, in the ΔpilR mutant. (d) PilR phosphorylation activates HSAF biosynthesis operon expression, as measured by RT-qPCR. lafB, the first gene of the operon. (e) PilR-dependent changes in LchD protein abundance, as measured by western blot. The plasmid-borne lchD-FLAG gene driven by its native promoter was introduced in the strains described in Figure 3c, and protein abundance was detected by the anti-FLAG antibody. The RecA protein was used as a loading control. (f) HSAF production, as quantified by high performance liquid chromatography. (g) HSAF production, as quantified by liquid broth (the semisolid medium without agar). Therefore, genetic evidence suggests that Clp binding enhances the DGC activity of LchD. Lastly, we wanted to investigate the fate of the LchD–Clp complex after c-di-GMP loading of Clp. Because biochemical experiments were deemed highly challenging due to the inability to purify full-length LchD, we measured LchD–Clp interactions in a heterologous host, E. coli. To accomplish this, we performed the B2H assay in two E. coli strains, one of which (XL1-Blue) has low and another (XL1-Blue ΔpilR) high intracellular c-di-GMP concentrations. Cyclic di-GMP concentration in the ΔpilR strain is high because it lacks YhjH/PdeH, a dominant PDE (El Mouali et al., 2017; Fang et al., 2014; Hengge et al., 2016). The B2H assay detected LchD–Clp interactions only in the low c-di-GMP strain (Figure 5g), suggesting that high levels of c-di-GMP inhibit LchD–Clp interactions. This conclusion was confirmed by MST, where binding affinity of the cytoplasmic LchD-His to Clp-GST was found to be approximately fourfold lower in the presence of 2 µM c-di-GMP compared to no c-di-GMP. An analog of c-di-GMP, c-di-AMP, at 2 µM concentration, had negligible effect on the LchD–Clp interaction (Figure 5h), verifying the specificity of the effect of c-di-GMP. It is worth noting that the 2 µM c-di-GMP concentration used in the MST experiment is within the presumed physiological range, because the K_d of Clp–c-di-GMP binding is 1.5 µM (Xu et al., 2018). These results suggest loading of Clp with c-di-GMP probably results in dissociation of the LchD–Clp complex.
FIGURE 4  Genetic analysis of the PilR–LchD interaction. (a–d) Restoration of the defects of the ΔpilR mutation by the lchD deletion, ΔlchD. Tests were performed in the ΔpilR mutant containing an additional deletion in either lchD (ΔpilR ΔlchD) or gene encoding a different diguanylate cyclase (DGC). (a) HSAF production, as quantified by high performance liquid chromatography (HPLC). (b) lofB mRNA abundance measured by quantitative reverse transcription PCR. (c) Plate antifungal assay. (d) HSAF production quantified by the plate antifungal assay. (e–h) Restoration of the pilR deletion defects by inactivation of the DGC activity of LchD via a chromosomal lchDGGAAV mutation. (e) HSAF production (quantified by HPLC) in the wild-type background (OH11): pBBR, empty vector; slr1143, gene encoding a potent DGC. (f) Plate antifungal assay. OH11(lchD) and OH11(lchDGGAAV), the wild-type OH11 containing a plasmid-borne lchD or its variation by substitution of GGDEV motif to GGAAV, respectively. (g) HSAF production quantified by the plate antifungal assay. (h) HSAF production in the ΔpilR mutant background. In (a, b, d, e, g, h), average data ± SD from three experiments are presented. **p < .01
activity (Figures 2f and S2). The affinity of DNA binding by PilR is stimulated by phosphorylation by its cognate histidine kinase, PilS (Figure 3a–c). Decreased LchD protein abundance (Figure 3e) contributes to low c-di-GMP synthesis, which in turn is necessary for the HSAF biosynthesis operon expression. While PilR decreases the levels of the LchD protein only moderately (Figure 3e), the consequences on HSAF production are strong (Figure 3f–h), probably because LchD directly interacts with Clp (Figure 5a–f). The ability of LchD to load Clp with c-di-GMP at the cytoplasmic membrane increases the ratio of c-di-GMP-loaded to c-di-GMP-free Clp, thus inhibiting operon expression and HSAF levels (Figure 7). However, incomplete (c.70%) restoration of the ΔpilR phenotype by the ΔlchD deletion points to the existence of yet another pathway through with PilR affects HSAF production (Figure 4).
LchD is a new addition to the network of c-di-GMP synthesizing and degrading enzymes involved in HSAF biosynthesis. This list includes the DGC WspR and two PDEs, LchP and RpfG (Xu et al., 2018, 2021). We recently showed that WspR directly interacts with the c-di-GMP-binding coactivator CdgL (YajQ), which together with the transcription factor LysR activates the HSAF biosynthesis operon (Han et al., 2020; Xu et al., 2021). Our finding that the second known c-di-GMP effector in L. enzymogenes, Clp, has a different dedicated DGC presents a striking example of nonoverlapping c-di-GMP signal transduction cascades regulating a common target, HSAF biosynthesis operon expression. This example strengthens the emerging paradigm of high specificity in c-di-GMP signaling, where dedicated DGCs and PDEs respond to unique environmental or intracellular stimuli and transmit those signals through specific c-di-GMP effectors (Dahlstrom et al., 2015; Giacalone et al., 2018; Hobley et al., 2012; Xu et al., 2018; Yang et al., 2017).

Earlier we reported that Clp also specifically interacts with the PDE LchP (Xu et al., 2018). It is therefore tempting to propose that LchD and LchP form a membrane-bound signaling complex dedicated to loading and unloading Clp (but not CdgL) with c-di-GMP. It is peculiar that Clp binding appears to enhance the DGC activity of LchD, while after loading with c-di-GMP, Clp–c-di-GMP disengages from LchD (Figure 6). Detailed characterization of the LchD–LchP–Clp signaling complex is a subject of ongoing research.

At present, we do not know what signals control the DGC activity of LchD or the PDE activity of LchP. Based on the domain architecture, LchD is positioned to respond to several stimuli through its periplasmic domain and/or the 7-transmembrane region. LchP also contains several periplasmic and cytoplasmic sensory domains.
Because HSAF is a key factor allowing L. enzymogenes to inhibit fungal growth (Figures 3g and 4c,f) and to prey on fungal cells, it is possible that some of the signals are fungal metabolites. Neither do we know what stimulates the PilS–PilR two-component system in L. enzymogenes. However, in P. aeruginosa, the PilS kinase senses abundance of the main T4P subunit, PilA (Kilmury & Burrows, 2016). Given that L. enzymogenes PilS is similar to its P. aeruginosa homolog in domain architecture and sequence, it is possible that L. enzymogenes PilS responds to the same stimulus. In this scenario, low levels of pilin would activate the PilS–PilR cascade, leading to simultaneous increase in the synthesis of T4P pili and HSAF. Coordinated up-regulation of motility and production of a “long-distance” antifungal weapon may facilitate bacterial grazing of soil and plant surfaces being unimpeded by fungal competitors, or grazing the territory covered with killed fungal cells, which are a food source for L. enzymogenes. Preying on fungal cells requires several hydrolytic enzymes, and L. enzymogenes is a master producer of such enzymes (hence the species name). Interestingly, production of these enzymes is co-regulated with HSAF, being mediated by Clp (Kobayashi et al., 2005; Qian et al., 2009); therefore, surface motility in L. enzymogenes is coordinated by PilR with synthesis of a fungal antibiotic (HSAF) and digestive enzymes.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains, plasmids, and growth conditions

The strains and plasmids used in this study are shown in Table S1. E. coli strains were grown in lysogenic broth (LB) medium at 37 °C with appropriate antibiotics. Unless otherwise specified, L. enzymogenes was grown in LB medium at 28 °C. The following antibiotics were used: kanamycin (Km), 30 μg/ml, for mutant construction, and gentamicin (Gm), 150 μg/ml, for plasmid maintenance.

4.2 | Genetic methods

Double-crossover homologous recombination was used to generate in-frame deletion in L. enzymogenes OH11, as described previously (Qian, et al., 2012). Primers used for DNA amplification are shown in Table S2. Briefly, two regions flanking the gene of interest were amplified by PCR and cloned in the suicide vector pEX18Gm (Table S1). The final constructs were transformed in the wild-type strain by electroporation. The single-crossover

FIGURE 7 Model of the PilS–PilR-controlled LchD–c-di-GMP–Clp regulatory cascade affecting HSAF biosynthesis in Lysobacter enzymogenes. (a) Activation of PilS (dark lightning symbol) results in autophosphorylation and phosphotransfer to PilR. The phosphorylated PilR binds to the pilA promoter activating type IV pili-mediated twitching motility. In addition, PilR binds upstream of the lchD promoter and down-regulates lchD expression. Decreased levels of LchD shift the ratio c-di-GMP-loaded versus c-di-GMP-free Clp toward the latter. Clp binds to two sites (PA and PB) upstream of the HSAF biosynthesis operon and activates transcription, which leads to higher HSAF production. (b) In the absence of the stimulus for PilS, the unphosphorylated PilR binds to the lchD promoter poorly. The uninhibited lchD transcription results in higher LchD abundance, higher c-di-GMP synthesis, and more efficient loading of Clp with c-di-GMP. Clp–c-di-GMP fails to activate expression of the HSAF biosynthesis operon, resulting in low HSAF production.

(Figure 1). Because HSAF is a key factor allowing L. enzymogenes to inhibit fungal growth (Figures 3g and 4c,f) and to prey on fungal cells, it is possible that some of the signals are fungal metabolites. Neither do we know what stimulates the PilS–PilR two-component system in L. enzymogenes. However, in P. aeruginosa, the PilS kinase senses abundance of the main T4P subunit, PilA (Kilmury & Burrows, 2016). Given that L. enzymogenes PilS is similar to its P. aeruginosa homolog in domain architecture and sequence, it is possible that L. enzymogenes PilS responds to the same stimulus. In this scenario, low levels of pilin would activate the PilS–PilR cascade, leading to simultaneous increase in the synthesis of T4P pili and HSAF. Coordinated up-regulation of motility and production of a "long-distance" antifungal weapon may facilitate bacterial grazing of soil and plant surfaces being unimpeded by fungal competitors, or grazing the territory covered with killed fungal cells, which are a food source for L. enzymogenes. Preying on fungal cells requires several hydrolytic enzymes, and L. enzymogenes is a master producer of such enzymes (hence the species name). Interestingly, production of these enzymes is co-regulated with HSAF, being mediated by Clp (Kobayashi et al., 2005; Qian et al., 2009); therefore, surface motility in L. enzymogenes is coordinated by PilR with synthesis of a fungal antibiotic (HSAF) and digestive enzymes.
recombinants were selected on the LB agar supplemented with Km and Gm. The recombinants were cultured in LB without antibiotics for 6 hr and plated on LB agar containing 1/10 (wt/vol) sucrose and Km. The in-frame gene deletions resulted from the double-crossover recombination were verified by PCR using the primers listed in Table S2.

Double-crossover homologous recombination was also used to generate chromosomal gene insertions using primers listed in Table S2. In brief, intact genes or genes containing point mutations were amplified by PCR, cloned in the suicide vector pEX18Gm, followed by transformation in the wild type or deletion mutants by electroporation. Selection was done as described above.

Overexpression strains were generated as described previously (Qian et al., 2014). In brief, genes containing native promoter regions were PCR-amplified and cloned in the broad host range vector, pBBR1-MCS5 (Table S1).

### 4.3 HSAF Extraction and Quantification

HSAF was extracted as described earlier (Qian et al., 2013). Briefly, 20 ml of culture of the wild-type strain or its derivatives was grown in 1/10 tryptic soy broth (TSB) for 24 hr at 28 °C with shaking. After centrifugation, cell-free supernatants were collected and mixed with an equal volume of ethyl acetate. Following shaking for 1 hr, the ethyl acetate phase was collected and evaporated to dryness. The HSAF-containing residue was dissolved in 200 μl of methanol and subjected to HPLC. Relative amounts of HSAF are calculated as peak areas on the HPLC chromatograms normalized to culture OD<sub>600 nm</sub>.

### 4.4 Quantitative reverse transcription-PCR

*L. enzymogenes* was grown in 1/10 TSB and cells were collected at OD<sub>600 nm</sub> 1.0. Total RNA was extracted using the Bacterial RNA Kit (OMEGA) according to the manufacturer’s protocol. RNA concentrations were measured by NanoDrop spectrophotometer ND-1000 UV (Thermo Fisher). RNA (400 ng) from each sample was used to generate total cDNA by the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa). RT-qPCR was performed in the Applied Biosystems 7500 machine. Primers for qPCR are listed in Table S2. The 16S rRNA gene was used as an internal control, as described previously (Xu et al., 2021). Each qPCR (20 μl) contained 10 μl of SYBR Premix ExTaq (TaKaRa), 2 × 0.4 μl primer, 0.4 μl ROX dye II (TaKaRa), 2 μl cDNA, and 6.8 μl water. The cycling programme was: 95 °C for 30 s, then 40 cycles of 95 °C for 5 s, and 60 °C for 34 s. The amplified products were detected by SYBR Green fluorescence. Amplification specificity was evaluated by melting curve analysis, where the melting curve was generated using 1 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 v. 2.0.6 software. Relative fold change in expression was calculated by the 2<sup>-ΔΔCT</sup> method (Livak & Schmittgen, 2001).

### 4.5 Antifungal activity assay

Plate antifungal assay was performed as described previously (Yang, Ren, Shen, Chou, et al., 2020). In brief, 4 μl of crude extract of HSAF from the wild-type OH11 and its derivatives was spotted at the edge of 1/10 TSB agar plates inoculated at the plate centre with *V. pyri* strain SXYL134 (Table S1), which was transferred from potato dextrose agar. After 2 days of incubation at 26 °C, the antifungal activity was measured as the inhibition zone around the spotted crude extract. The lengths of the longest axis and the shortest axis were averaged and considered as radius. The area was calculated as π × (radius)<sup>2</sup>.

### 4.6 *E. coli* reporter assays for evaluating DGC activity

The *E. coli*-based motility inhibition was used to monitor DGC activity, as reported earlier (Xu et al., 2018). Briefly, swim zones in semi-solid agar formed by the highly motile strain *E. coli* MG1655 serve as an indicator of intracellular c-di-GMP concentration. Expression of heterologous DGCs from the plasmid-borne vector result in smaller swim zones (Ryu et al., 2017; Xu et al., 2018). The constructs expressing the full-length IChD and its mutant derivatives were cloned in the vector pBAD/Myc-His B (Table S1) and transformed into *E. coli* MG1655. The transformed *E. coli* strains were grown in LB for 12 hr, and 3 μl of culture was spotted onto soft (0.25%) agar plates containing 1% tryptone, 0.5% NaCl with or without 0.1% arabinose. Diameters of the swim zones were measured after 8 hr incubation at 37 °C.

### 4.7 Bacterial one-hybrid and two-hybrid assays

The bacterial one-hybrid system (Agilent Technologies) was used to detect protein–DNA interactions, as described elsewhere (Guo et al., 2009; Xu et al., 2016). In brief, the bait promoter fragments were cloned in vector of pBXcmT (Table S1) upstream of the selectable reporter genes, HIS3 and adaA. The target protein was expressed as a fusion to the N-terminal domain of the α-subunit of RNA polymerase in vector pTRG. The reporter genes were expressed in *E. coli* XL1-Blue MRF<sup>+</sup> Kan (Table S1) only when the target protein binds to the promoter region. Transcription of the HIS3 reporter gene results in the synthesis of imidazolesglycerol-phosphate dehydratase, which enables colony growth in the presence of the competitive inhibitor 3-amino-1,2,4-triazole (3-AT). Transcription of the second reporter gene, adaA, encoding a protein conferring streptomycin (Str) resistance, validates protein–DNA interactions. All transformants were grown on the selective medium (+3AT + Str) for 2 days at 28 °C. The selective medium is minimal M9 medium amended with 5 mM 3-AT, 2 μg/ml Str, 12.5 μg/ml tetracycline, 34 μg/ml chloramphenicol, and 30 μg/ml Km, as described previously (Xu et al., 2016). Plasmids pBX-R2031 and pTRG-R3133 were used as positive control, while
empty vectors, pTRG and pBXcmT, were used as negative control (Guo et al., 2009).

The bacterial two-hybrid system (Agilent Technologies) was used to detect protein–protein interactions as described in Xu et al. (2021). In brief, the coding DNA sequences of the target genes were cloned into pBT and pTRG and then cotransformed into E. coli XL1-Blue MRF³ Kan or its Δythl mutant (Table S1). All transformants were grown on the same selective medium (+3AT + Str) as described in the B1H assay for 2 days at 28 °C. If there was a direct physical interaction between LchD and its partner, the transformed E. coli containing both plasmids would be able to grow well on the selective medium. Plasmids pBT-GacS and pTRG-GacS were used as a positive control; empty vectors pTRG and pBT were used as negative control.

4.8 | Protein expression and purification

The LchD and Clp proteins were expressed as His-tagged and purified by affinity chromatography. The coding regions of the lchD and clp genes were cloned in plasmid pET30a (Table S1) using the primers listed in Table S2. The clp gene with also cloned in plasmid pOP-THisLip (Table S1). The genes were expressed in E. coli BL21(DE3) (Table S1). The His-fusion proteins were purified using the Ni-NTA resin (GE Healthcare) from 400 ml of E. coli BL21(DE3) carrying the pET30a plasmid derivatives. The strains were grown at 37 °C to OD600 nm = 0.5, after which gene expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma) for 4 hr at 28 °C. The concentration of purified proteins was determined by the BCA protein assay kit (Sangon Biotech). Protein purity was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Expression and purification of GST-Clp, GST-CdgL, and PilR-His were described recently (Chen et al., 2017; Han et al., 2020; Xu et al., 2018; Yang, Ren, Shen, Yang, et al., 2020).

4.9 | Electrophoretic mobility shift assay

EMSA was performed as described previously (Ge et al., 2020; Yang, Ren, Shen, Yang, et al., 2020). The 5-carboxy-fluorescein (FAM)-labelled probes comprising the promoter region upstream of lchD were synthesized by GENEWIZ. The labelled DNA fragments (100 ng) were mixed and incubated with various concentrations of purified PilR-His at 25 °C for 20 min in an EMSA/Gel-Shift Binding Buffer (Beyotime). The mixtures were then loaded onto an 8% polyacrylamide gel and electrophoresed for 1 hr. The labelled DNA fragments were detected using a VersaDoc imaging system (Bio-Rad).

4.10 | Microscale thermophoresis assay

Binding of the PilR-His protein to promoter fragments labelled with FAM was determined by MST assay using Monolith NT.115 (NanoTemper Technologies), according to the procedure described previously (Han et al., 2020; Xu et al., 2018). A constant concentration (10 μM) of the labelled promoters in the MST buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 0.05% Tween 20) was titrated against increasing concentrations of PilR-His dissolved in double-distilled water.

The protein–protein binding affinities were determined by MST using Monolith NT.115 as described previously (Su et al., 2017; Xu et al., 2018). In brief, the LchD-His protein was labelled with the fluorescent dye RED-Tris-NTA (NanoTemper Technologies) via amine conjugation. A constant concentration (100 nM) of the labelled promoter in the MST buffer was titrated against Clp-GST protein or CdgL-GST protein (concentration range 0.38 nM to 25 μM). MST premium-coated capillaries (Monolith NT.115 MO-K005) were used to load the samples into the MST instrument at 25 °C using high MST power and 60% LED power. Laser on and off times were set at 30 and 5 s, respectively. All experiments were conducted in triplicate. Data were analysed using NanoTemper Analysis v. 1.2.101 software (NanoTemper Technologies).

4.11 | Pull-down assays

Pull-down assays were performed as described previously (Yang, Ren, Shen, Yang, et al., 2020). The reaction mixtures contained 5 μM of LchD-His and Clp-GST or CdgL-GST in 1 ml of phosphate-buffered saline (PBS). Then 50 μl of GST resin was added and samples were incubated at 4 °C overnight. The resin was collected by centrifugation at 4 °C (500 × g, 5 min) and washed three times with PBS containing 1% Triton X-100 to remove nonspecifically bound proteins. Proteins captured on the GST-beads were eluted by boiling in 4 × SDS loading dye for 6 min, after which samples were subjected to SDS-PAGE and western blotting. Protein detection involved GST- (ab19256) and His- (ab18184) specific antibodies (Abcam).

4.12 | Reproducibility and statistical analysis

Experiments were performed three times, with three technical replicates in each experiment. Statistical analyses were carried out using Student’s t test (α = 0.05) implemented in the SPSS v. 14.0 package (SPSS Inc.).

ACKNOWLEDGEMENTS

This study was supported by the National Natural Science Foundation of China (32072470 and 31872016 to G.Q.), the Natural Science Foundation of Jiangsu Province (BK20190026 and BK20181325 to G.Q.), Fundamental Research Funds for the Central Universities (KJJQ202001, KYT201805 and KYT2201403 to G.Q.), and the Innovation Team Program for Jiangsu Universities (2017 to G.Q.). The funders had no role in the study design.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.
AUTHOR CONTRIBUTIONS
G.Q. conceived the project. G.Q., S.C., and M.G. designed the experiments. K.X., D.S., and N.Y. carried out experiments. K.X., G.Q., S.C., and M.G. analysed data and prepared figures and tables. G.Q. and K.X. wrote the manuscript draft. G.Q., S.C., and M.G. revised the manuscript. All authors read and approved the submission for publication.

DATA AVAILABILITY STATEMENT
The sequence data from the present study have been submitted to the NCBI GenBank at https://www.ncbi.nlm.nih.gov/genbank/ under the following accession numbers: MG387198.1 (Le0082), MG387192.1 (Le0155), MG387199.1 (Le0901), MG387200.1 (Le1158), MG387201.1 (Le2120), MG387202.1 (Le2826), MG387206.1 (Le3756; LchD), MG387196.1 (Le3882), MG387209.1 (Le4562; WspR), MG387213.1 (Le4875), MG387193.1 (Le2762; LchP) and MG387215.1 (Le4727; RpFg). All other data needed to evaluate the conclusions in the paper are presented in the paper or in the supporting information.

ORCID
Guoliang Qian https://orcid.org/0000-0003-3577-3241

REFERENCES
Baraquet, C. & Harwood, C.S. (2016) FleQ DNA binding consensus sequence revealed by studies of FleQ-dependent regulation of biofilm gene expression in Pseudomonas aeruginosa. Journal of Bacteriology, 198, 178–186. https://doi.org/10.1128/JB.00539-15
Baraquet, C., Murakami, K., Parsek, M.R. & Harwood, C.S. (2012) The FleQ protein from Pseudomonas aeruginosa functions as both a repressor and an activator to control gene expression from the pel operon promoter in response to c-di-GMP. Nucleic Acids Research, 40, 7207–7218. https://doi.org/10.1093/nar/gks384
Bellini, D., Caly, D.L., McCarthy, Y., Bumann, M., An, S.Q., Dow, J.M., et al. (2014) Crystal structure of an HD-GYP domain cyclic-dGMP phosphodiesterase reveals an enzyme with a novel trnucleic catalytic iron centre. Molecular Microbiology, 91, 26–38. https://doi.org/10.1111/mmi.12447
Boyd, J.M., Koga, T. & Lory, S. (1994) Identification and characterization of PilS, an essential regulator of pilin expression in Pseudomonas aeruginosa. Molecular and General Genetics, 243, 565–574. https://doi.org/10.1007/BF00284205
Chen, Y., Xia, J., Su, Z., Xu, G., Komelsky, M., Qian, G. et al. (2017) Lysobacter PilR, the regulator of type IV pilus synthesis, controls antifungal antibiotic production via a cyclic di-GMP pathway. Applied and Environmental Microbiology, 83, e03397. https://doi.org/10.1128/AEM.03397-16
Christen, M., Christen, B., Folcher, M., Schauerte, A. & Jenal, U. (2005) Identification and characterization of a cyclic di-GMP-specific phosphodiesterase and its allosteric control by GTP. The Journal of Biological Chemistry, 280, 30829–30837. https://doi.org/10.1074/jbc.M504429200
Christensen, P. & Cook, F.D. (1978) Lysobacter, a new genus of nonfruiting, gliding bacteria with a high base ratio. International Journal of Systematic and Evolutionary Microbiology, 28, 367–393. https://doi.org/10.1099/00207713-28-3-367
Dahlstrom, K.M., Giglio, K.M., Collins, A.J., Sondermann, H. & O'Toole, G.A. (2015) Contribution of physical interactions to signaling specificity between a diguanylate cyclase and its effector. mBio, 6, e01978. https://doi.org/10.1128/mBio.01978-15
El Mouali, Y., Kim, H., Ahmad, I., Brauner, A., Liu, Y., Skurnik, M. et al. (2017) Stand-alone EAL domain proteins form a distinct subclass of EAL proteins involved in regulation of cell motility and biofilm formation in Enterobacteria. Journal of Bacteriology, 199, e00179. https://doi.org/10.1128/JB.00179-17
El-Gebali, S., Mistry, J., Bateman, A., Eddy, S.R., Luciani, A., Potter, S.C. et al. (2019) The Pfam protein families database in 2019. Nucleic Acids Research, 47, D427–D432. https://doi.org/10.1093/nar/gky995
Fang, X., Ahmad, I., Blanka, A., Schottkowski, M., Cimadis, A., Galperin, M.Y. et al. (2014) GIL, a new c-di-GMP-binding protein domain involved in regulation of cellulose synthesis in enterobacteria. Molecular Microbiology, 93, 439–452. https://doi.org/10.1111/mmi.12672
Ge, M., Wang, Y., Liu, Y., Jiang, L.U., He, B., Ning, L. et al. (2020) The NIN-like protein 5 (ZmNLPS) transcription factor is involved in modulating the nitrogen response in maize. The Plant Journal, 102, 353–368. https://doi.org/10.1111/tpj.14628
Giacalone, D., Smith, T.J., Collins, A.J., Sondermann, H., Koziol, L.J. & O'Toole, G.A. (2018) Ligand-mediated biofilm formation via enhanced physical interaction between a diguanylate cyclase and its receptor. mBio, 9, e01254. https://doi.org/10.1128/mBio.01254-18
Guo, M., Feng, H., Zhang, J., Wang, W., Wang, Y., Li, Y. et al. (2009) Dissecting transcription regulatory pathways through a new bacterial one-hybrid reporter system. Genome Research, 19, 1301–1308. https://doi.org/10.1101/gr.086595.108
Han, S., Shen, D.Y., Wang, Y.C., Chou, S.H., Gemelsky, M., Gao, Y.G. et al. (2020) A YajQ-LysR-like, cyclic di-GMP-dependent system regulating biosynthesis of an antifungal antibiotic in a crop-protecting bacterium, Lysobacter enzymogenes. Molecular Plant Pathology, 21, 218–229. https://doi.org/10.1111/mpp.12890
Han, Y., Wang, Y., Tombosa, S., Wright, S., Hoffman, J., Yuen, G. et al. (2015) Identification of a small molecule signaling factor that regulates the biosynthesis of the antifungal polycyclic tetramate macrolactam HSAF in Lysobacter enzymogenes. Applied Microbiology and Biotechnology, 99, 801–811. https://doi.org/10.1007/s00253-014-6120-x
Hengge, R., Galperin, M.Y., Ghigo, J.M., Gemelsky, M., Green, J., Hughes, K.T. et al. (2016) Systematic nomenclature for GGDEF and EAL domain-containing cyclic-di-GMP turnover proteins of Escherichia coli. Journal of Bacteriology, 198, 7–11. https://doi.org/10.1128/JB.00424-15
Hernández-Eliogó, A., Andrade, Á., Soto, L., Morett, E. & Juárez, K. (2017) The unphosphorylated form of the PilR two-component system regulates pilA gene expression in Geobacter sulfurreducens. Environmental Science and Pollution Research International, 24, 25693–25701. https://doi.org/10.1007/s11356-016-6192-5
Hickman, J.W. & Harwood, C.S. (2008) Identification of FleQ from Pseudomonas aeruginosa as a c-di-GMP-responsive transcription factor. Molecular Microbiology, 69, 376–389. https://doi.org/10.1111/j.1365-2958.2008.06281.x
Hickman, J.W., Tibrea, D.F. & Harwood, C.S. (2005) A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. Proceedings of the National Academy of Sciences of the United States of America, 102, 14422–14427. https://doi.org/10.1073/pnas.0507170102
Hobbs, M., Collie, E.S., Free, P.D., Livingston, S.P. & Mattick, J.S. (1993) PilS and PilR, a two-component transcriptional regulatory system controlling expression of type 4 fimbriae in Pseudomonas aeruginosa. Molecular Microbiology, 7, 669–682. https://doi.org/10.1111/j.1365-2958.1993.tb01158.x
Hobley, L., Fung, R.K.Y., Lambert, C., Harris, M.A.T.S., Dahbi, J.M., King, S.S. et al. (2012) Discrete cyclic-diGMP-dependent control of bacterial predation versus axenic growth in Bdellovibrio...
bacteriovorus. PLoS Pathogens, 8, e1002493. https://doi.org/10.1371/journal.ppat.1002493

Ishimoto, K.S. & Lory, S. (1992) Identification of pilR, which encodes a transcriptional activator of the Pseudomonas aeruginosa pilin gene. Journal of Bacteriology, 174, 3514–3521. https://doi.org/10.1128/JB.174.11.3514-3521.1992

Jain, R., Sliusarenko, O. & Kazmierczak, B.I. (2017) Interaction of the cyclic-di-GMP binding protein FixM and the Type IV pilus assembly ATPase promotes pilus assembly. PLoS Pathogens, 13, e1006594. https://doi.org/10.1371/journal.ppat.1006594

Jin, S., Ishimoto, K.S. & Lory, S. (1994) PilR, a transcriptional regulator of pilusion in Pseudomonas aeruginosa, binds to a cis-acting sequence upstream of the pilin gene promoter. Molecular Microbiology, 14, 1049–1057. https://doi.org/10.1111/j.1365-2958.1994.tb01338.x

Kehl-Fie, T.E., Porsch, E.A., Miller, S.E. & St Geme, J.W. (2009) Expression of Kingella kingae type IV pili is regulated by sigma54, PilS, and PilR. Journal of Bacteriology, 191, 4976–4986. https://doi.org/10.1128/JB.00123-09

Kilmury, S.L. & Burrows, L.L. (2016) Type IV pilins regulate their own expression via direct intramembrane interactions with the sensor kinase PilS. Proceedings of the National Academy of Sciences of the United States of America, 113, 6017–6022. https://doi.org/10.1073/pnas.1512947113

Kilmury, S.L.N. & Burrows, L.L. (2018) The Pseudomonas aeruginosa PilSR two-component system regulates both twitching and swimming motilities. mbio, 9, e01310. https://doi.org/10.1128/mBio.01310-18

Kobayashi, D.Y., Reedy, R.M., Palumbo, J.D., Zhou, J.M. & Yuen, G.Y. (2005) A pil gene homologue belonging to the Crp gene family globally regulates lytic enzyme production, antimicrobial activity, and biological control activity expressed by Lysobacter enzymogenes strain C3. Applied and Environmental Microbiology, 71, 261–269. https://doi.org/10.1128/AEM.71.1.261-269.2005

Li, S.J., Du, L.C., Yuen, G. & Harris, S.D. (2006) Distinct ceramides synthesize polarized growth in the filamentous fungus Aspergillus nidulans. Molecular Biology of the Cell, 17, 1218–1227. https://doi.org/10.1091/mbc.e05-06-0533

Lin, L., Zhou, M., Shen, D., Han, S., Fulano, A.M., Chou, S.-H. et al. (2020) A non-flagellated biocontrol bacterium employs a PilZ-PilB complex to provoke twitching motility associated with its predation behavior. Phytopathology Research, 2, 12. https://doi.org/10.1101/s42483-020-00054-x

Livak, K.J. & Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-△△Ct method. Methods, 25, 402–408. https://doi.org/10.1016/S1215-6773(01)00323-1

McCleary, W.R. & Stock, J.B. (1994) Acetyl phosphate and the activator of piliation in Myxococcus xanthus. Journal of Bacteriology, 174, 1792–1798. https://doi.org/10.1128/JB.174.5.1792-1798.2005

Meyer, R., Schmidt, A., Ryjenkov, D.A. & Gomelsky, M. (2005) The ubiquitin-proteasome pathway to the biosynthesis of a unique antifungal metabolite. Applied and Environmental Microbiology, 71, 261–269. https://doi.org/10.1128/AEM.71.1.261-269.2005

Ren, X., Ren, S., Xu, G., Dou, W., Chou, S.-H., Chen, Y.-U. et al. (2020) Knockout of diguanylate cyclase genes in Lysobacter enzymogenes to improve production of antifungal factor and increase its application in seed coating. Current Microbiology, 77, 1006–1015. https://doi.org/10.1007/s00284-020-01902-x

Römling, U., Galperin, M.Y. & Gomelsky, M. (2013) Cyclic di-GMP: The first 25 years of a universal bacterial second messenger. Microbiology and Molecular Biology Reviews, 77, 1–52. https://doi.org/10.1128/MMBR.00043-12

Ryu, M.-H., Fomicheva, A., O’Neill, L., Alexandre, G. & Gomelsky, M. (2017) Using light-activated enzymes for modulating intracellular c-di-GMP levels in bacteria. Methods in Molecular Biology, 1657, 169–186. https://doi.org/10.1007/978-1-4939-7240-1_14

Schmidt, A.J., Ryjenkov, D.A. & Gomelsky, M. (2005) Cyclic diguanylate is a ubiquitous signaling molecule in bacteria: Insights into biochemistry of the GGDEF protein domain. Journal of Bacteriology, 187, 1792–1798. https://doi.org/10.1128/JB.187.5.1792-1798.2005

Suzuki, H., Takahashi, Y., Ohsawa, K., Endo, T., Nakamura, Y. et al. (2018) 4-Hydroxybenzoic acid is a diffusible factor that connects metabolic shikimate pathway to the biosynthesis of a unique antifungal metabolite in Lysobacter enzymogenes. Molecular Microbiology, 104, 163–178. https://doi.org/10.1111/mmi.13619

Ryu, M.-H., Fomicheva, A., O’Neill, L., Alexandre, G. & Gomelsky, M. (2017) Using light-activated enzymes for modulating intracellular c-di-GMP levels in bacteria. Methods in Molecular Biology, 1657, 169–186. https://doi.org/10.1007/978-1-4939-7240-1_14

Seidel, S.A.I., Dijkman, P.M., Lea, W.A., van den Bogaart, G., Jerabek-Willemsen, M., Laiz, A. et al. (2013) Microscale thermophoresis quantifies biomolecular interactions under previously challenging conditions. Methods, 59, 301–315. https://doi.org/10.1016/j.ymeth.2012.12.005

Su, Z.H., Chen, H.F., Wang, P., Tombosa, S., Du, L.C., Han, Y. et al. (2017) The PilRS domain protein VIEa is a cyclic diguanylate phosphodiesterase. The Journal of Biological Chemistry, 280, 33324–33330. https://doi.org/10.1074/jbc.M116500200

Wang, Y., Zhao, Y., Zhang, J., Zhao, Y., Shen, Y., Su, Z. et al. (2014) Transcriptomic analysis reveals new regulatory roles of Clp signaling in secondary metabolite biosynthesis and surface motility in Lysobacter enzymogenes OH11. Applied Microbiology and Biotechnology, 98, 9009–9020. https://doi.org/10.1007/s00253-014-6072-1

Wu, S.S. & Kaiser, D. (1997) Regulation of expression of the pilA gene in Myxococcus xanthus. Journal of Bacteriology, 179, 7748–7758. https://doi.org/10.1128/jb.179.4.7748-7758.1997

Cell physiology and surface motility in Myxobacterium xanthus. Journal of Bacteriology, 179, 7748–7758. https://doi.org/10.1128/jb.179.4.7748-7758.1997
Xu, G.G., Han, S., Huo, C.M., Chin, K.H., Chou, S.H., Gomelsky, M. et al. (2018) Signaling specificity in the c-di-GMP-dependent network regulating antibiotic synthesis in Lysobacter. Nucleic Acids Research, 46, 9276–9288. https://doi.org/10.1093/nar/gky803

Xu, H.Y., Chen, H.F., Shen, Y.M., Du, L.C., Chou, S.H., Liu, H.X. et al. (2016) Direct regulation of extracellular chitinase production by the transcription factor LeClp in Lysobacter enzymogenes OH11. Phytopathology, 106, 971–977. https://doi.org/10.1094/PHYTO-01-16-0001-R

Xu, K.W., Shen, D.Y., Han, S., Chou, S.H. & Qian, G.L. (2021) A non-flagellated, predatory soil bacterium reprograms a chemosensory system to control antifungal antibiotic production via cyclic di-GMP signalling. Environmental Microbiology, 23(2), 878–892. https://doi.org/10.1111/1462-2920.15191

Yang, C.X., Cui, C.Y., Ye, Q.M., Kan, J.H., Fu, S.N., Song, S.H. et al. (2017) Burkholderia cenocepacia integrates cis-2-dodecenoic acid and cyclic dimeric guanosine monophosphate signals to control virulence. Proceedings of the National Academy of Sciences of the United States of America, 114, 13006–13011. https://doi.org/10.1073/pnas.1709048114

Yang, M.M., Ren, S.S., Shen, D.Y., Chou, S.H. & Qian, G.L. (2020) ClpP mediates antagonistic interaction of Lysobacter enzymogenes with a crop fungal pathogen. Biological Control, 140, 104125. https://doi.org/10.1016/j.biocontrol.2019.104125

Yang, M.M., Ren, S.S., Shen, D.Y., Yang, N.D., Wang, B.X., Han, S. et al. (2020) An intrinsic mechanism for coordinated production of the contact-dependent and contact-independent weapon systems in a soil bacterium. PLoS Pathogens, 16, e1008967. https://doi.org/10.1371/journal.ppat.1008967

Yu, F.A., Zaleta-Rivera, K., Zhu, X.C., Huffman, J., Millet, J.C., Harris, S.D. et al. (2007) Structure and biosynthesis of heat-stable antifungal factor (HSAF), a broad-spectrum antymycotic with a novel mode of action. Antimicrobial Agents and Chemotherapy, 51, 64–72. https://doi.org/10.1128/AAC.00931-06

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Xu K, Shen D, Yang N, Chou S-H, Gomelsky M, Qian G. Coordinated control of the type IV pili and c-di-GMP-dependent antifungal antibiotic production in Lysobacter by the response regulator PilR. Mol Plant Pathol. 2021;22:602–617. https://doi.org/10.1111/mpp.13046