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Lysobacter PilR, the Regulator of Type IV Pilus Synthesis, Controls Antifungal Antibiotic Production via a Cyclic di-GMP Pathway

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ABSTRACT *Lysobacter enzymogenes* is a ubiquitous soil gammaproteobacterium that produces a broad-spectrum antifungal antibiotic, known as heat-stable antifungal factor (HSAF). To increase HSAF production for use against fungal crop diseases, it is important to understand how HSAF synthesis is regulated. To gain insights into transcriptional regulation of the HSAF synthesis gene cluster, we generated a library with deletion mutations in the genes predicted to encode response regulators of the two-component signaling systems in *L. enzymogenes* strain OH11. By quantifying HSAF production levels in the 45 constructed mutants, we identified two strains that produced significantly smaller amounts of HSAF. One of the mutations affected a gene encoding a conserved bacterial response regulator, PilR, which is commonly associated with type IV pilus synthesis. We determined that *L. enzymogenes* PilR regulates pilus synthesis and twitching motility via a traditional pathway, by binding to the *pilA* promoter and upregulating *pilA* expression. Regulation of HSAF production by PilR was found to be independent of pilus formation. We discovered that the *pilR* mutant contained significantly higher intracellular levels of the second messenger cyclic di-GMP (c-di-GMP) and that this was the inhibitory signal for HSAF production. Therefore, the type IV pilus regulator PilR in *L. enzymogenes* activates twitching motility while downregulating antibiotic HSAF production by increasing intracellular c-di-GMP levels. This study identifies a new role of a common pilus regulator in proteobacteria and provides guidance for increasing antifungal antibiotic production in *L. enzymogenes*.

IMPORTANCE PilR is a widespread response regulator of the two-component system known for regulating type IV pilus synthesis in proteobacteria. Here we report that, in the soil bacterium *Lysobacter enzymogenes*, PilR regulates pilus synthesis and twitching motility, as expected. Unexpectedly, PilR was also found to control intracellular levels of the second messenger c-di-GMP, which in turn inhibits production of the antifungal antibiotic HSAF. The coordinated production of type IV pili and antifungal antibiotics has not been observed previously.

KEYWORDS antibiotics, HSAF, *Lysobacter*, PilR, type IV pili

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The genus *Lysobacter*, belonging to the family *Xanthomonadaceae*, is ubiquitous in the environment (1). Among more than 30 described *Lysobacter* species, *Lysobacter enzymogenes* is the best studied (2, 3). Two *L. enzymogenes* strains, C3 and OH11, produce antifungal antibiotics, which are applied to control crop fungal diseases (4–6). One antibiotic, i.e., heat-stable antifungal factor (HSAF), a polycyclic tetramate macro-

lactam with a distinct chemical structure, has broad-spectrum antifungal activity (7, 8). It is synthesized via a unique biosynthetic pathway, in which a hybrid polyketide synthase and a nonribosomal peptide synthetase, encoded by the *lafB* gene (originally described as *hsaf pks/nrps*), within the HSAF biosynthesis cluster catalyze the linkage of ornithine to two polyketides (9, 10). HSAF inhibits fungal pathogens by targeting sphingolipid biosynthesis, which is a distinct target, compared to the targets of other antifungal agents (11), thus making HSAF particularly attractive for antifungal control.

Understanding the mechanisms regulating HSAF biosynthesis in *L. enzymogenes* is important for the purpose of increasing antibiotic production. Some initial insights into HSAF regulation have been obtained; however, the regulatory picture is far from complete. We and our collaborators have shown that HSAF levels are increased when *L. enzymogenes* is grown in poorer medium, e.g., 0.1× tryptic soy broth (TSB), compared to regular 1× TSB (8, 11, 12). This observation suggests that HSAF synthesis depends on extracellular stimuli. In support of this hypothesis, two two-component systems (TCSs) that affect HSAF biosynthesis in *L. enzymogenes* have been identified (12–14). One of these TCSs, i.e., RpfC-RpfG, activates HSAF production in response to extracellular levels of the fatty acid signaling molecule diffusible signaling factor 3 (DSF3) (12, 13). Another member of a TCS family, PilG, which is an orphan response regulator (RR) protein, was found to negatively regulate HSAF biosynthesis in response to as yet unknown stimuli (14).

According to our genomic survey, strain *L. enzymogenes* OH11 encodes 48 putative histidine kinases (HKs) and 53 RRs (Fig. 1). We hypothesized that some of the remaining TCSs in *L. enzymogenes* might also be involved in regulating HSAF biosynthesis. To analyze the roles of these remaining TCSs, we decided to knock out each RR gene. As a result, we generated a genome-wide library of the in-frame RR deletion mutants in *L. enzymogenes*. By screening this deletion library, we unexpectedly found that PilR, the RR associated with regulation of type IV pilus (T4P) genes, is involved in regulating HSAF production. Here we show that PilR is a *bona fide* regulator of T4P synthesis and twitching motility in *L. enzymogenes* and that it regulates HSAF independently of T4P. Our findings suggest that the PilS-PilR TCS affects HSAF production via the cyclic di-GMP (c-di-GMP) signaling pathway, with c-di-GMP being a ubiquitous bacterial second messenger (15). In addition to the discovery of a new TCS involved in HSAF regulation and its unexpected role in controlling c-di-GMP signaling, our study has uncovered an antagonistic relationship between twitching motility and antibiotic production in *L. enzymogenes*.

RESULTS

Generation and analysis of the RR deletion library in *L. enzymogenes*. To investigate the potential role of *L. enzymogenes* TCSs in HSAF production, we analyzed the genome of strain OH11 for the presence of TCSs. Using the Pfam database, we identified 48 putative HKs and 53 putative RRs, which represent 41 paired HK-RR TCSs and 19 orphan TCSs (7 HKs and 12 RRs) (Fig. 1; also see Table S1 in the supplemental material). As expected, the RRs fell into three categories, based on their output domains. Group I, which harbors RRs with only receiver domains and no identifiable output domains, has 6 representatives in *L. enzymogenes*. Group II contains 42 representatives, each of which has an N-terminal receiver domain linked to a C-terminal DNA-binding domain. Group III contains 5 RRs that possess N-terminal receiver domains attached to C-terminal domains with various enzymatic activities, most of which contain GGDEF, EAL, or HD-GYP domains involved in c-di-GMP synthesis or hydrolysis (16).

We generated a deletion mutant library with each of the remaining RR-encoding genes. Forty-five genes were individually deleted. Genes encoding six RRs (Le0736, Le0752, Le2296, Le3679, Le4789, and Le4845) could not to be deleted despite several attempts, which suggests that these RRs are potentially essential for bacterial survival under our experimental conditions. We compared the growth rates of the generated RR mutants in the medium for maximal HSAF production (0.1× TSB) and found that none

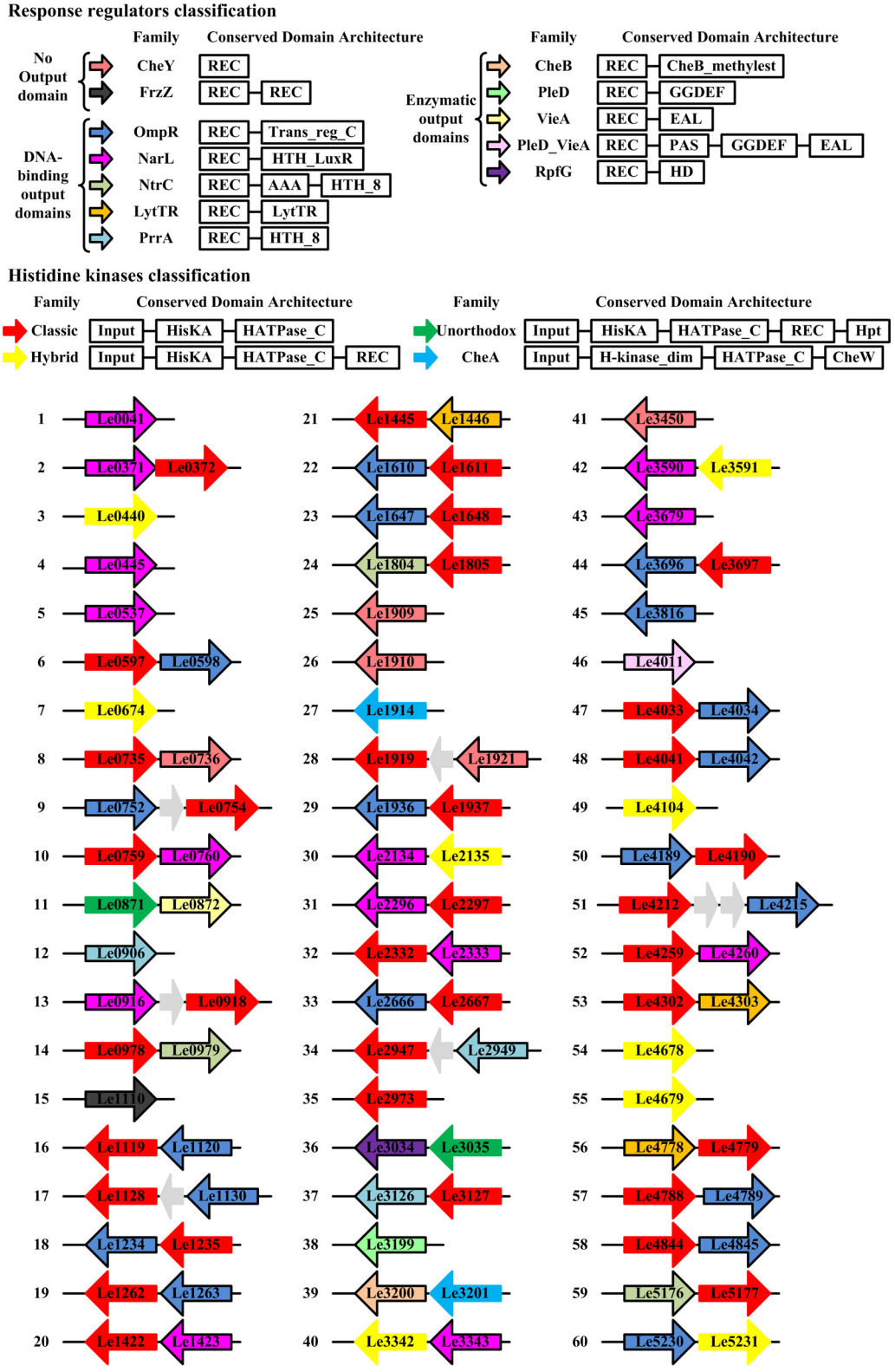

FIG 1 Identification of two-component systems (TCSs) in *L. enzymogenes* OH11. The histidine kinases (HKs) and response regulators (RRs) were classified according to the P2CS database (40). HKs and RRs belonging to various families are depicted in different colors.

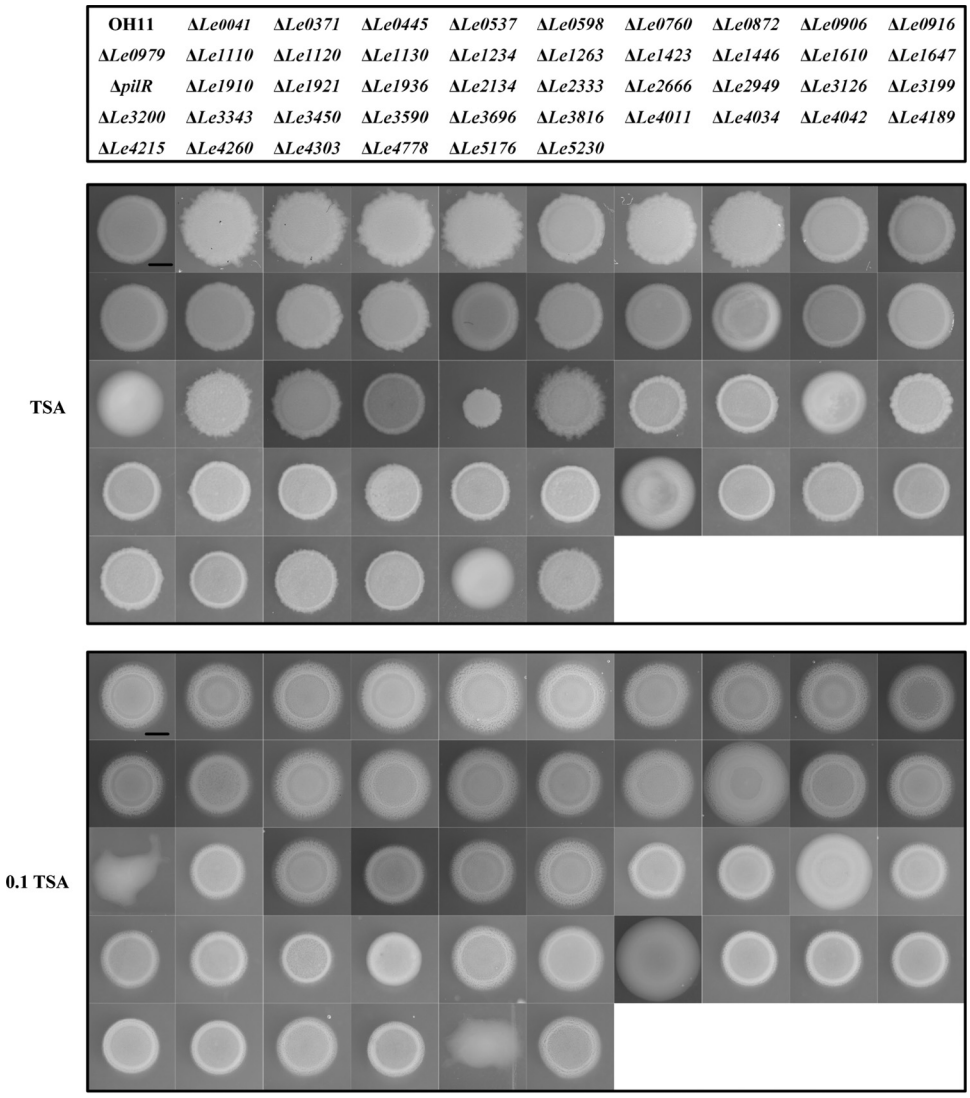

FIG 2 *L. enzymogenes* RR deletion mutants displaying no significant growth defects in HSAF-inducing medium. TSA is the nutrient-rich medium used as the control, and 0.1 TSA is the HSAF-inducing medium. Scale bar, 2 mm. The growth curves of each mutant in liquid 0.1× TSB are shown in Fig. S1 in the supplemental material.

of the mutants showed significant growth defects, compared to the wild-type strain, although several mutants had different colony morphologies, compared to the wild-type strain (Fig. 2; Fig. S1).

We quantified HSAF production in each RR mutant by high-performance liquid chromatography (HPLC). Two RR proteins (RpfG and PilG) were known to control HSAF levels, based on our earlier work (13, 14). In the present work, we used the *rpfG* deletion mutant (Δ*rpfG*) as a control and confirmed that HSAF levels were significantly decreased in the mutant. In addition, we found two new mutants, with mutations in the *pilR* and *Le3200* genes, that exhibited significant reductions in HSAF levels, compared to the wild-type strain (Fig. 3A; Table S2).

Indirect activation of HSAF biosynthesis by PilR. In this study, we focused on one of the newly found RRs involved in HSAF synthesis regulation, namely, PilR; Le3200 will be subject to a separate study. PilR belongs to the PilS-PilR TCS, which is conserved in proteobacteria and is involved in the regulation of T4P synthesis and twitching motility (17–19). This TCS also plays a role in bacterial attachment to surfaces and biofilm formation (20–23).

To ascertain the role of PilR in the regulation of HSAF biosynthesis, we complemented the *pilR* mutant with plasmid-borne *pilR*. The complemented strain produced

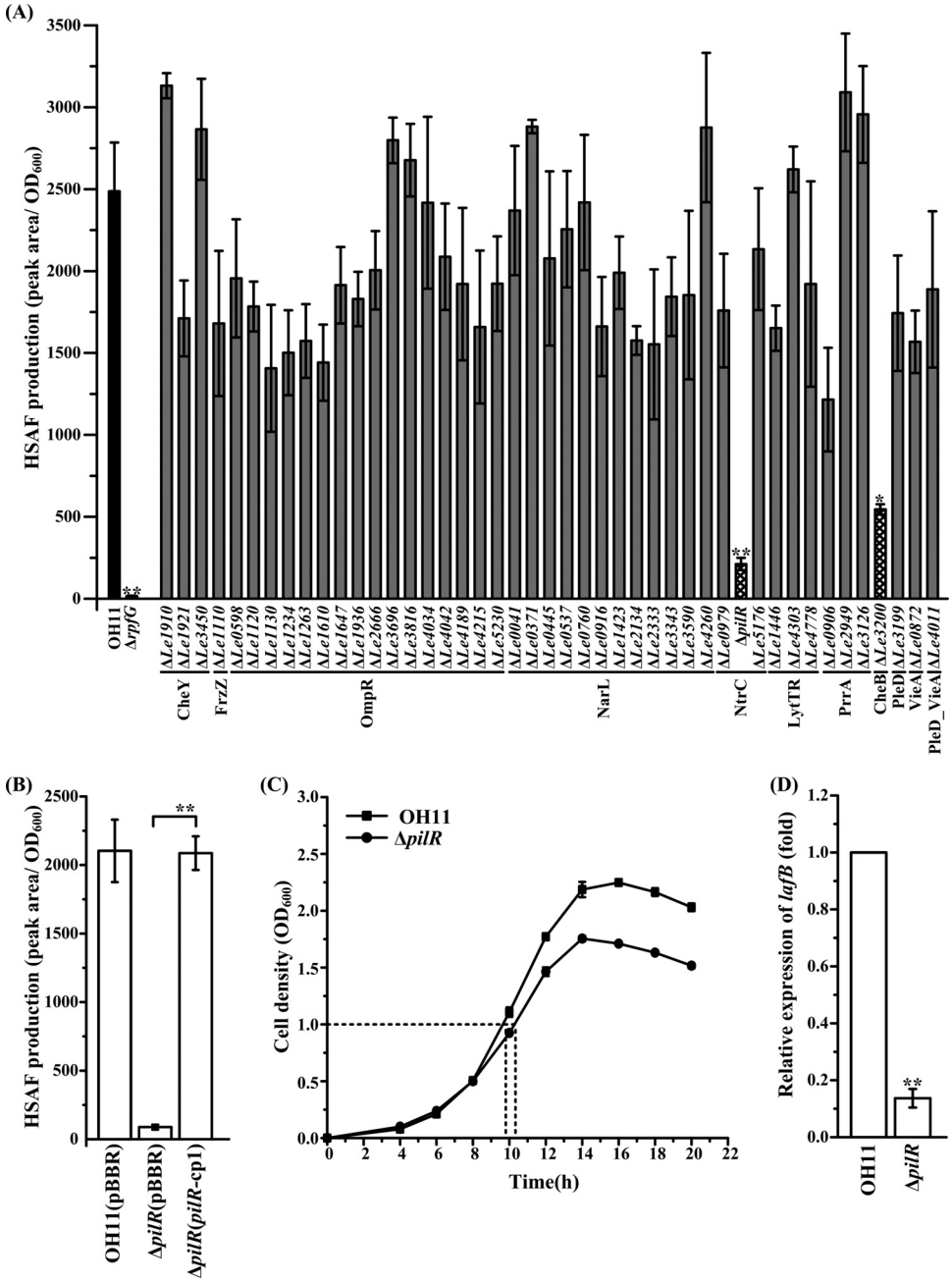

FIG 3 Quantification of HSAF produced by the *L. enzymogenes* RR mutants. (A) HSAF production, measured by HPLC and normalized to OD_{600} values. Data from triplicate experiments are shown. *, $P < 0.05$; **, $P < 0.01$. (B) Complementation of the Δ*pilR* mutant with the plasmid-borne *pilR* gene, rescuing HSAF production. Error bars represent standard deviations. Δ*pilR*(pBBR), the *pilR* mutant carrying an empty vector (pBBR1-MCS5); Δ*pilR*(*pilR*-cp1), the *pilR* mutant with plasmid *pilR*-pBBR, carrying the intact *pilR* gene. **, $P < 0.01$. (C) Representative growth curves of wild-type and Δ*pilR* strains in the HSAF-inducing medium (0.1× TSB). The dashed lines indicate the time points at which cells reached an OD_{600} of 1.0, when they were collected for qRT-PCR analysis, as shown in panel D. (D) qRT-PCR analysis of *lafB* mRNA levels. The *lafB* mRNA level in the wild-type strain OH11 was set as 1. **, $P < 0.01$.

similar amounts of HSAF, compared to the wild-type strain (Fig. 3B). To investigate the level at which PilR affects HSAF production, we measured the levels of the transcript of *lafB* (originally described as *hsaf pks/nrps*), the key HSAF biosynthetic gene (9). Results of the quantitative reverse transcription (qRT)-PCR analysis showed that *lafB* mRNA levels were significantly lower in the *pilR* mutant, compared to the wild-type strain (Fig. 3C and D), which suggests that PilR regulates HSAF biosynthesis at the level of gene expression.

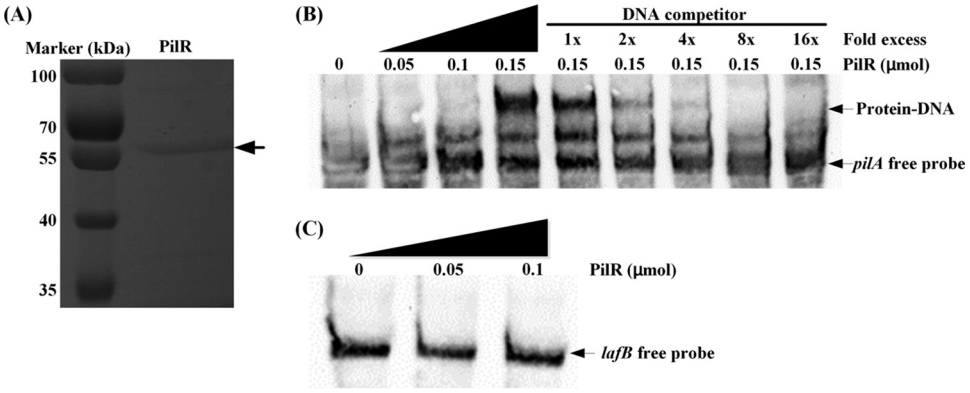

FIG 4 EMSA showing binding of PilR to the *pilA* promoter but not the *lafB* promoter. (A) SDS-PAGE analysis of the purified His-tagged PilR. (B) PilR binding to the *pilA* promoter *in vitro*. The unlabeled *pilA* probe provided in excess with respect to the labeled probe competitively inhibits formation of the PilR-DNA complex. Arrows indicate free DNA and PilR-DNA complexes. (C) No detection of PilR binding to the *lafB* promoter.

Next, we tested the ability of PilR to bind to the *lafB* promoter, using an electrophoretic mobility shift assay (EMSA). To this end, we overexpressed and purified PilR as a His_6-fusion (Fig. 4A). As a positive control, we used the 541-bp promoter region upstream of *L. enzymogenes pilA*, which was chosen on the basis of the previously characterized PilR-regulated *pilA* promoter from *Pseudomonas aeruginosa* (24). The EMSA revealed the PilR-DNA complex with the *L. enzymogenes pilA* probe (Fig. 4B). This complex could be competitively inhibited by excess unlabeled *pilA* probe, which suggests that the interactions are specific (Fig. 4B). Under similar conditions, however, no protein-DNA complex was observed between PilR and the *lafB* promoter (Fig. 4C), which suggests that PilR affects HSAF biosynthesis gene expression indirectly.

PilS-PilR TCS in *L. enzymogenes* regulation of T4P-driven twitching motility. Since PilR was found to bind to the *L. enzymogenes pilA* promoter, we expected it to be involved in the formation of T4P-driven twitching motility (14, 25). Consistent with this expectation, the *pilR* mutant produced no motile cells that could migrate away from the margin of the colony, which is in contrast to the wild-type strain (Fig. 5A). The impairment of the *pilR* mutant in twitching motility could be rescued by the *pilR*-expressing plasmid but not the empty vector (Fig. 5A). Furthermore, the *pilA* mRNA levels were greatly downregulated in the Δ*pilR* strain, compared to the wild-type strain (Fig. 5B and C). These results demonstrate that *L. enzymogenes* PilR acts as a *bona fide* regulator of T4P synthesis and twitching motility.

To test whether the *L. enzymogenes* PilS, the predicted HK of PilR, acts upstream of PilR in the same signal transduction cascade, we created an in-frame deletion in the *pilS* gene (Table 1). HSAF quantification and twitching motility tests showed that the *pilS* deletion caused a significant drop in HSAF production (Fig. 6A) and complete loss of twitching motility (Fig. 6B). These results are consistent with the key role of the PilS-PilR TCS in coordinate regulation of twitching motility and HSAF biosynthesis in *L. enzymogenes*.

PilR regulation of HSAF biosynthesis via a c-di-GMP signaling pathway. Since PilR regulates HSAF gene transcription indirectly, we turned to the transcription factor Clp, which was identified by us earlier as a major contributor to HSAF gene expression (25). We hypothesized that PilR may act upstream of Clp. The Clp proteins from *Xanthomonas* species, which are closely related to *Lysobacter*, bind c-di-GMP and sense intracellular c-di-GMP levels (26). Therefore, it is possible that *L. enzymogenes* PilR affects either *clp* gene expression or c-di-GMP levels. Our proteomics data suggest that the levels of the Clp protein in the *pilR* mutant and the wild-type strain do not significantly differ (Fig. S2); therefore, we looked at the potential role of PilR in changing c-di-GMP levels.

Prior to exploring the PilR-c-di-GMP link, we wanted to test whether intracellular c-di-GMP levels play any role in HSAF production. To this end, we introduced into *L.*

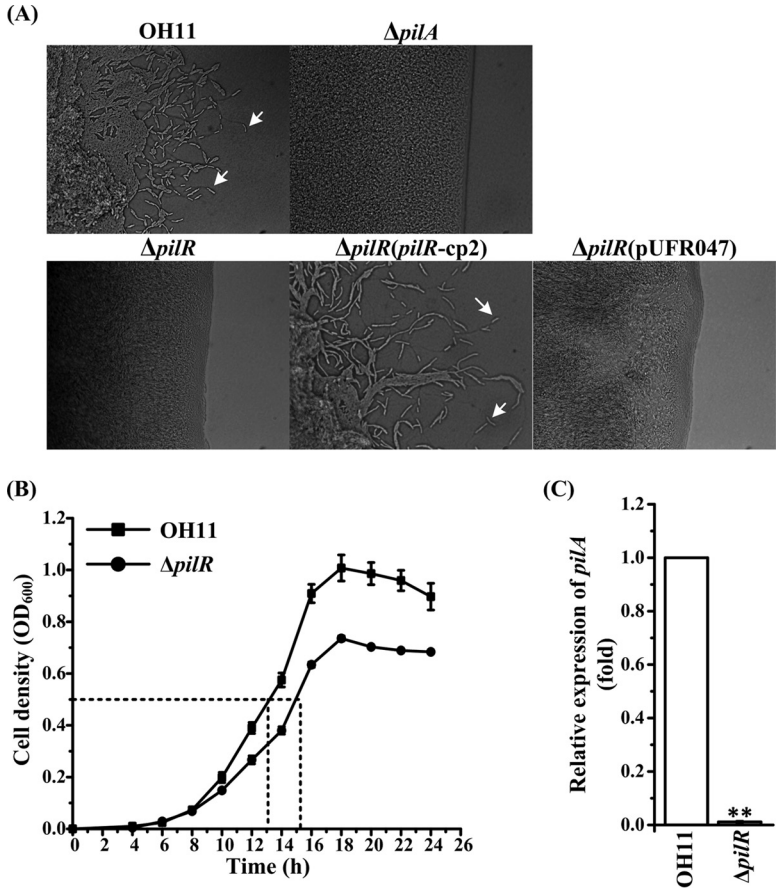

FIG 5 PilR involvement in regulating twitching motility in *L. enzymogenes*. (A) Indicated by arrows are motile cells at the margin of a colony, which is characteristic of twitching motility in *L. enzymogenes* (25). Δ*pilR*(pURF047), the Δ*pilR* mutant containing an empty vector; Δ*pilR*(*pilR*-cp2), the Δ*pilR* mutant with plasmid *pilR*-pUFR047, containing the intact *pilR* gene. Δ*pilA*, the strain lacking T4P and deficient in twitching motility (25), was used as a control. (B) Growth curves of the wild-type strain and the Δ*pilR* mutant in 0.05× TSB (the medium optimal for twitching motility) (25). The dashed lines indicate cells at an OD_{600} of 0.5, which were collected for qRT-PCR analysis, as shown in panel C. (C) qRT-PCR analysis of *pilA* mRNA in the wild-type and Δ*pilR* strains. The *pilA* mRNA level in the wild-type OH11 strain was set as 1. **, $P < 0.01$. Three replicates were used for each treatment, and the experiment was performed three times.

enzymogenes a potent diguanylate cyclase (c-di-GMP synthase), i.e., Slr1143 from *Synechocystis* sp., and a potent c-di-GMP phosphodiesterase, i.e., YhjH (PdeH) from *Escherichia coli* (27, 28). The *slr1143* and *yhjH* genes were constitutively expressed from the broad-host-range vectors (Table 1). As shown in Fig. 7A, introduction of the phosphodiesterase gene *yhjH* into the *pilR* mutant caused a significant increase in the HSAF yield, while introduction of the diguanylate cyclase gene *slr1143* slightly decreased the HSAF yield (Fig. 7A). These findings suggest that lower HSAF production in the *pilR* mutant may have been caused by elevated c-di-GMP levels. To test this prediction, we measured, by liquid chromatography-mass spectrometry (LC-MS), the intracellular c-di-GMP levels in the wild-type strain and the Δ*pilR* strain. In accord with our expectations, the intracellular c-di-GMP levels in the *pilR* mutant were significantly elevated, compared to the levels in the wild-type strain (Fig. 7B). To gain an additional piece of evidence indicating that elevated c-di-GMP levels are inhibitory to HSAF production, we introduced the plasmid-borne *slr1143* gene into the wild-type strain and found that HSAF production was significantly decreased (Fig. S3). These results strongly suggest that elevated c-di-GMP levels are inhibitory for HSAF production and that PilR regulates HSAF biosynthesis via a c-di-GMP signaling pathway.

Because several PilR-type transcription regulators, e.g., FleQ from *P. aeruginosa* (29, 30) and XbmR from *Xanthomonas citri* (31), have been shown to bind c-di-GMP directly,

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Source
Strains		
Lysobacter enzymogenes		
Wild-type		
OH11	Wild-type; Km^r	6
In-frame deletion mutants		
Δ*Le0041*	In-frame deletion of *Le0041*; Km^r	This study
Δ*Le0371*	In-frame deletion of *Le0371*; Km^r	This study
Δ*Le0445*	In-frame deletion of *Le0445*; Km^r	This study
Δ*Le0537*	In-frame deletion of *Le0537*; Km^r	This study
Δ*Le0598*	In-frame deletion of *Le0598*; Km^r	This study
Δ*Le0760*	In-frame deletion of *Le0760*; Km^r	This study
Δ*Le0872*	In-frame deletion of *Le0872*; Km^r	This study
Δ*Le0906*	In-frame deletion of *Le0906*; Km^r	This study
Δ*Le0916*	In-frame deletion of *Le0916*; Km^r	This study
Δ*Le0979*	In-frame deletion of *Le0979*; Km^r	This study
Δ*Le1110*	In-frame deletion of *Le1110*; Km^r	This study
Δ*Le1120*	In-frame deletion of *Le1120*; Km^r	This study
Δ*Le1130*	In-frame deletion of *Le1130*; Km^r	This study
Δ*Le1234*	In-frame deletion of *Le1234*; Km^r	This study
Δ*Le1263*	In-frame deletion of *Le1263*; Km^r	This study
Δ*Le1423*	In-frame deletion of *Le1423*; Km^r	This study
Δ*Le1446*	In-frame deletion of *Le1446*; Km^r	This study
Δ*Le1610*	In-frame deletion of *Le1610*; Km^r	This study
Δ*Le1647*	In-frame deletion of *Le1647*; Km^r	This study
Δ*pilR*	In-frame deletion of *pilR*; Km^r	This study
Δ*Le1910*	In-frame deletion of *Le1910*; Km^r	This study
Δ*Le1921*	In-frame deletion of *Le1921*; Km^r	This study
Δ*Le1936*	In-frame deletion of *Le1936*; Km^r	This study
Δ*Le2134*	In-frame deletion of *Le2134*; Km^r	This study
Δ*Le2333*	In-frame deletion of *Le2333*; Km^r	This study
Δ*Le2666*	In-frame deletion of *Le2666*; Km^r	This study
Δ*Le2949*	In-frame deletion of *Le2949*; Km^r	This study
Δ*Le3126*	In-frame deletion of *Le3126*; Km^r	This study
Δ*Le3199*	In-frame deletion of *Le3199*; Km^r	This study
Δ*Le3200*	In-frame deletion of *Le3200*; Km^r	This study
Δ*Le3343*	In-frame deletion of *Le3343*; Km^r	This study
Δ*Le3450*	In-frame deletion of *Le3450*; Km^r	This study
Δ*Le3590*	In-frame deletion of *Le3590*; Km^r	This study
Δ*Le3696*	In-frame deletion of *Le3696*; Km^r	This study
Δ*Le3816*	In-frame deletion of *Le3816*; Km^r	This study
Δ*Le4011*	In-frame deletion of *Le4011*; Km^r	This study
Δ*Le4034*	In-frame deletion of *Le4034*; Km^r	This study
Δ*Le4042*	In-frame deletion of *Le4042*; Km^r	This study
Δ*Le4189*	In-frame deletion of *Le4189*; Km^r	This study
Δ*Le4215*	In-frame deletion of *Le4215*; Km^r	This study
Δ*Le4260*	In-frame deletion of *Le4260*; Km^r	This study
Δ*Le4303*	In-frame deletion of *Le4303*; Km^r	This study
Δ*Le4778*	In-frame deletion of *Le4778*; Km^r	This study
Δ*Le5176*	In-frame deletion of *Le5176*; Km^r	This study
Δ*Le5230*	In-frame deletion of *Le5230*; Km^r	This study
Δ*pilS*	In-frame deletion of *pilS*; Km^r	This study
Δ*rpfG*	In-frame deletion of *rpfG*; Km^r	13
Δ*pilR* Δ*pilA*	In-frame deletion of *pilR* and *pilA*; Km^r	This study
Δ*pilR* Δ*lafB*	In-frame deletion of *pilR* and *lafB*; Km^r	This study
Complementary strains		
OH11(pBBR)	OH11 harboring plasmid pBBR1-MCS5; Gm^r, Km^r	This study
Δ*pilR*(pBBR)	Δ*pilR* harboring plasmid pBBR1-MCS5; Gm^r, Km^r	This study
Δ*pilR* Δ*pilA*(pBBR)	Δ*pilR* Δ*pilA* harboring plasmid pBBR1-MCS5; Gm^r, Km^r	This study
Δ*pilR*(pUFR047)	Δ*pilR* harboring plasmid pUFR047; Ap^r, Gm^r, Km^r	This study
Δ*pilR* Δ*lafB*(pUFR047)	Δ*pilR* Δ*lafB* harboring plasmid pUFR047; Ap^r, Gm^r, Km^r	This study
Δ*pilR*(*pilR*-cp1)	Δ*pilR* harboring plasmid *pilR*-pBBR; Gm^r, Km^r	This study
Δ*pilR*(*pilR*-cp2)	Δ*pilR* harboring plasmid *pilR*-pUFR047; Ap^r, Gm^r, Km^r	This study
Δ*pilR* Δ*pilA*(*pilR*-cp1)	Δ*pilR* Δ*pilA* harboring plasmid *pilR*-pBBR; Gm^r, Km^r	This study
Δ*pilR* Δ*lafB*(*pilR*-cp2)	Δ*pilR* Δ*lafB* harboring plasmid *pilR*-pUFR047; Ap^r, Gm^r, Km^r	This study

(Continued on next page)

TABLE 1 (Continued)

Strain or plasmid	Characteristics[a]	Source
Δ*pilR*(*slr*-pBBR)	Δ*pilR* harboring plasmid *slr*-pBBR; Gm^r, Km^r	This study
Δ*pilR*(*yhjH*-pBBR)	Δ*pilR* harboring plasmid *yhjH*-pBBR; Gm^r, Km^r	This study
Escherichia coli		
DH5α	Host strain for molecular cloning	Laboratory collection
BL21(DE3)	Host strain for protein expression	Laboratory collection
Plasmids		
pEX18GM	Suicide vector with *sacB* gene; Gm^r	41
pBBR1-MCS5	Broad-host-range vector with P*lac* promoter	42
pUFR047	Low-copy-number plasmid; Gm^r, Ap^r	43
pET30a	Protein expression vector; Km^r	Novagen
slr-pBBR	pBBR1-MCS5 cloned with Gm promoter and 1,032-bp fragment containing intact *slr1143*; Gm^r	This study
yhjH-pBBR	pBBR1-MCS5 cloned with Gm promoter and 768-bp fragment containing intact *yhjH*; Gm^r	This study
0041-pEX18	pEX18GM with two flanking fragments of *Le0041*; Gm^r	This study
0371-pEX18	pEX18GM with two flanking fragments of *Le0041*; Gm^r	This study
0445-pEX18	pEX18GM with two flanking fragments of *Le0445*; Gm^r	This study
0537-pEX18	pEX18GM with two flanking fragments of *Le0537*; Gm^r	This study
0598-pEX18	pEX18GM with two flanking fragments of *Le0598*; Gm^r	This study
0760-pEX18	pEX18GM with two flanking fragments of *Le0760*; Gm^r	This study
0872-pEX18	pEX18GM with two flanking fragments of *Le0872*; Gm^r	This study
0906-pEX18	pEX18GM with two flanking fragments of *Le0906*; Gm^r	This study
0916-pEX18	pEX18GM with two flanking fragments of *Le0916*; Gm^r	This study
0979-pEX18	pEX18GM with two flanking fragments of *Le0979*; Gm^r	This study
1110-pEX18	pEX18GM with two flanking fragments of *Le1110*; Gm^r	This study
1120-pEX18	pEX18GM with two flanking fragments of *Le1120*; Gm^r	This study
1130-pEX18	pEX18GM with two flanking fragments of *Le1130*; Gm^r	This study
1234-pEX18	pEX18GM with two flanking fragments of *Le1234*; Gm^r	This study
1263-pEX18	pEX18GM with two flanking fragments of *Le1263*; Gm^r	This study
1423-pEX18	pEX18GM with two flanking fragments of *Le1423*; Gm^r	This study
1446-pEX18	pEX18GM with two flanking fragments of *Le1446*; Gm^r	This study
1610-pEX18	pEX18GM with two flanking fragments of *Le1610*; Gm^r	This study
1647-pEX18	pEX18GM with two flanking fragments of *Le1647*; Gm^r	This study
pilR-pEX18	pEX18GM with two flanking fragments of *pilR*; Gm^r	This study
1910-pEX18	pEX18GM with two flanking fragments of *Le1910*; Gm^r	This study
1921-pEX18	pEX18GM with two flanking fragments of *Le1921*; Gm^r	This study
1936-pEX18	pEX18GM with two flanking fragments of *Le1936*; Gm^r	This study
2134-pEX18	pEX18GM with two flanking fragments of *Le2134*; Gm^r	This study
2333-pEX18	pEX18GM with two flanking fragments of *Le2333*; Gm^r	This study
2666-pEX18	pEX18GM with two flanking fragments of *Le2666*; Gm^r	This study
2949-pEX18	pEX18GM with two flanking fragments of *Le2949*; Gm^r	This study
3126-pEX18	pEX18GM with two flanking fragments of *Le3126*; Gm^r	This study
3199-pEX18	pEX18GM with two flanking fragments of *Le3199*; Gm^r	This study
3200-pEX18	pEX18GM with two flanking fragments of *Le3200*; Gm^r	This study
3343-pEX18	pEX18GM with two flanking fragments of *Le3343*; Gm^r	This study
3450-pEX18	pEX18GM with two flanking fragments of *Le3450*; Gm^r	This study
3590-pEX18	pEX18GM with two flanking fragments of *Le3590*; Gm^r	This study
3696-pEX18	pEX18GM with two flanking fragments of *Le3696*; Gm^r	This study
3816-pEX18	pEX18GM with two flanking fragments of *Le3816*; Gm^r	This study
4011-pEX18	pEX18GM with two flanking fragments of *Le4011*; Gm^r	This study
4034-pEX18	pEX18GM with two flanking fragments of *Le4034*; Gm^r	This study
4042-pEX18	pEX18GM with two flanking fragments of *Le4042*; Gm^r	This study
4189-pEX18	pEX18GM with two flanking fragments of *Le4189*; Gm^r	This study
4215-pEX18	pEX18GM with two flanking fragments of *Le4215*; Gm^r	This study
4260-pEX18	pEX18GM with two flanking fragments of *Le4260*; Gm^r	This study
4303-pEX18	pEX18GM with two flanking fragments of *Le4303*; Gm^r	This study
4778-pEX18	pEX18GM with two flanking fragments of *Le4778*; Gm^r	This study
5176-pEX18	pEX18GM with two flanking fragments of *Le5176*; Gm^r	This study
5230-pEX18	pEX18GM with two flanking fragments of *Le5230*; Gm^r	This study
pilS-pEX18	pEX18GM with two flanking fragments of *pilS*; Gm^r	This study
pilR-pBBR	pBBR1-MCS5 cloned with 1,540-bp fragment containing intact *pilR*; Gm^r	This study
pilR-pUFR047	pUFR047 cloned with 1,540-bp fragment containing intact *pilR*; Ap^r, Gm^r	This study
pilR-pET30(a)	pET30a cloned with fragment containing full-length *pilR*; Km^r	This study

[a]Km^r, kanamycin resistant; Gm^r, gentamicin resistant; Ap^r, ampicillin resistant.

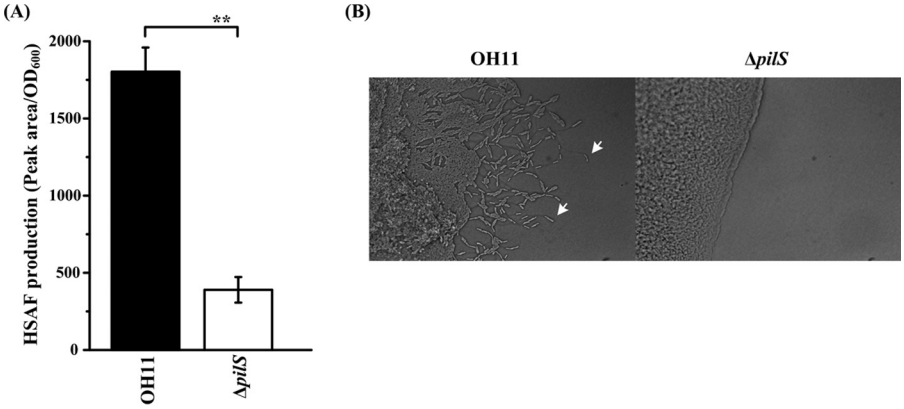

FIG 6 *L. enzymogenes* PilS involvement in regulating HSAF biosynthesis (A) and twitching motility (B). Three technical replicates were used for each treatment, and the biological experiment was performed three times. Vertical bars represent standard errors. **, $P < 0.01$, relative to the wild-type OH11 strain. Δ*pilS*, the *pilS* deletion mutant. Arrows indicate motile cells at the margins of a colony.

we also tested, using microscale thermophoresis, the ability of *L. enzymogenes* PilR to bind c-di-GMP. However, we found no evidence of c-di-GMP binding (Fig. S4).

Independence of regulation of HSAF biosynthesis and twitching motility by PilR. The results of the experiments described above suggest that *L. enzymogenes* PilR controls HSAF biosynthesis and twitching motility via two independent pathways. To verify this conclusion, we generated and tested two double mutants, i.e., Δ*pilR* Δ*pilA* and Δ*pilR* Δ*lafB*, which were impaired in motility and HSAF synthesis, respectively (Table 1). Then we introduced the plasmid-borne *pilR* gene into these double mutants and quantified HSAF production and motility. As shown in Fig. 8A, the Δ*pilR* Δ*pilA* double mutant lacking T4P was rescued with respect to HSAF production by the *pilR*-expressing plasmid, which shows that T4P are not involved in the PilR-dependent

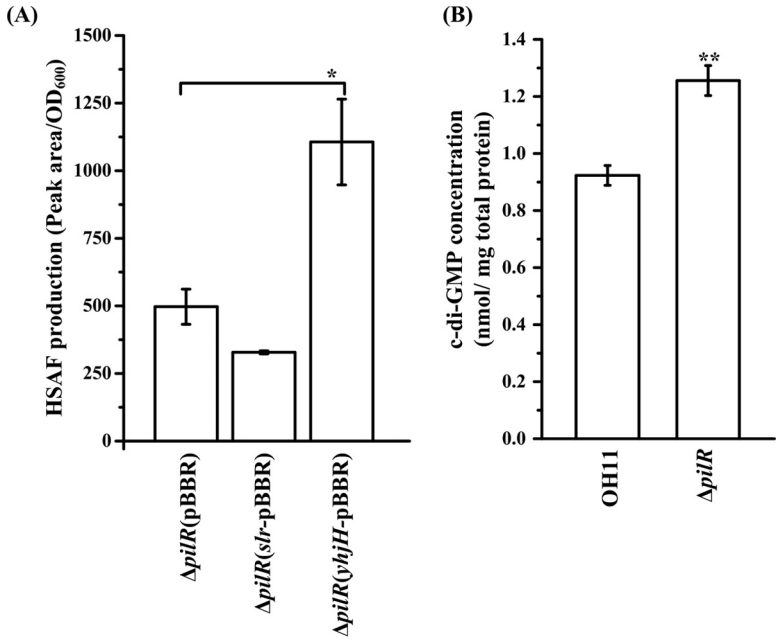

FIG 7 Intracellular c-di-GMP levels affecting HSAF production in the *pilR* mutant. (A) The c-di-GMP phosphodiesterase YhjH increased, while the diguanylate cyclase Slr1143 decreased, HSAF production in the Δ*pilR* mutant. Δ*pilR*(pBBR), Δ*pilR*(*slr*-pBBR), and Δ*pilR*(*yhjH*-pBBR) are *pilR* mutant strains containing an empty vector, the plasmid-borne *slr1143*, and *yjhH*, respectively. *, $P < 0.05$. (B) The *pilR* mutant had significantly elevated intracellular c-di-GMP levels. Three technical replicates were used for each treatment, and the biological experiment was performed three times. **, $P < 0.01$.

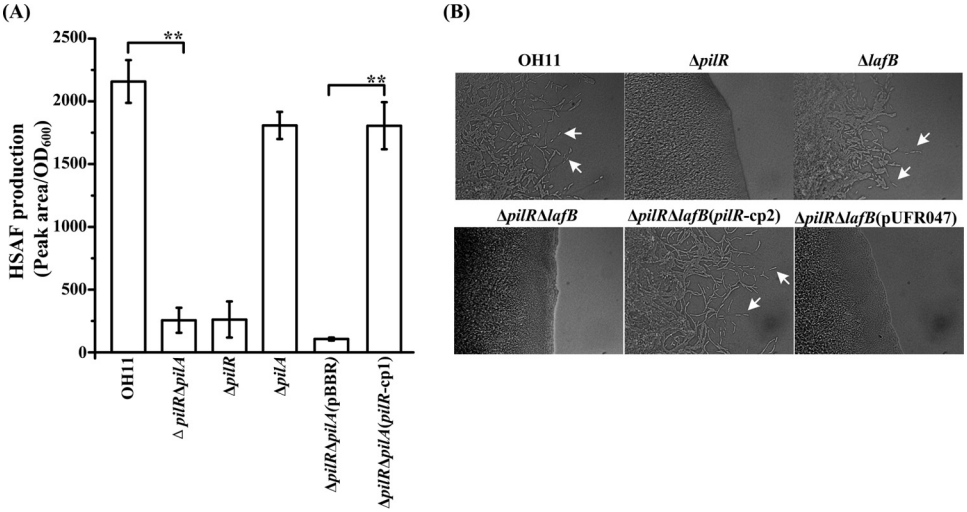

FIG 8 Independent PilR regulation of HSAF production and twitching motility. (A) HSAF quantification in the wild-type strain and mutants. Δ*pilR*Δ*pilA*(pBBR) and Δ*pilR*Δ*pilA*(*pilR*-cp1) indicate the Δ*pilR* Δ*pilA* double mutant containing an empty vector (pBBR1-MCS5) and the plasmid *pilR*-pBBR, carrying the intact *pilR* gene, respectively. **, $P < 0.01$. (B) Twitching motility of the Δ*pilR* Δ*lafB* double mutant containing an empty vector (pURF047) or plasmid *pilR*-pUFR047, carrying the intact *pilR* gene. Arrows indicate motile cells at the margins of a colony.

regulation of HSAF production. Similarly, twitching motility of the Δ*pilR* Δ*lafB* double mutant was fully restored by the *pilR*-expressing plasmid (Fig. 8B), which indicates that HSAF production does not affect motility. Furthermore, the Δ*pilA* mutant made with the wild-type genetic background produced HSAF levels similar to the levels of the wild-type strain (Fig. 8A), while the HSAF-deficient Δ*lafB* mutant was unaffected with respect to twitching motility, compared to the wild-type strain (Fig. 8B). Taken together, our results show that PilR coordinates T4P-driven twitching motility and HSAF production in *L. enzymogenes* via independent pathways (Fig. 9).

DISCUSSION

L. enzymogenes is a biocontrol bacterium that produces HSAF, a promising antifungal agent (7, 8). Because of its agricultural applications, *L. enzymogenes* is emerging as

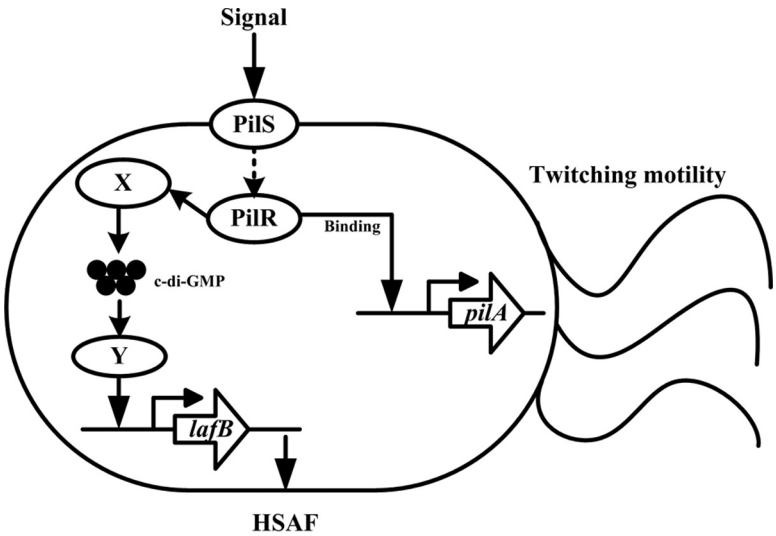

FIG 9 Proposed model of dual regulation by *L. enzymogenes* PilR. PilR forms a TCS with its cognate histidine kinase, PilS. Upon activation, PilR directly activates *pilA* transcription, which is required for T4P twitching motility. PilR affects the synthesis (or degradation) of c-di-GMP (system X) (indicated by an arrow), which in turn affects the activity of a transcription factor (Y) that regulates *lafB* gene expression and HSAF biosynthesis.

an important model for studying the regulation of HSAF biosynthesis. Previous studies identified several regulators of HSAF production (12, 14, 25, 32). To expand the range of potential factors affecting HSAF synthesis and to gain insights into the mechanisms of such regulation, we systematically deleted genes encoding RRs of TCSs in *L. enzymogenes*. We found two new regulators, i.e., PilR and Le3200, the latter of which will be characterized in a separate study.

Finding PilR as a regulator of HSAF production was unexpected, because PilR is a highly conserved RR of T4P synthesis and twitching motility in proteobacteria but it has not been known to affect secondary metabolite synthesis. In this study, we confirmed that, according to expectations, *L. enzymogenes* PilR functions as an activator of *pilA* expression and is required for twitching motility. We also showed that PilS, a cognate HK of PilR, acts in the expected manner. The PilR-mediated regulation of HSAF production turned out to be indirect and independent of the regulation of T4P genes. Because several NtrC-type RRs to which PilR belongs, including *P. aeruginosa* FleQ (29, 30) and *X. citri* XbmR (31), bind c-di-GMP directly, in this work we tested *L. enzymogenes* PilR for c-di-GMP binding; however, no binding was detected. Intriguingly, we found that the *pilR* deletion resulted in elevated intracellular c-di-GMP levels, which proved to be inhibitory for HSAF production. The latter conclusion was confirmed by our manipulation of c-di-GMP levels via heterologous diguanylate cyclase (Slr1143) and c-di-GMP phosphodiesterase (YhjH/PdeH). Our finding of c-di-GMP as an inhibitory stimulus for HSAF production suggests that a strain with a constitutive or induced system for decreasing c-di-GMP levels could show improved HSAF yields in industrial applications.

The mechanisms underlying the inhibitory role of c-di-GMP in HSAF gene expression remain to be explored. One candidate for mediating such regulation is Clp, whose requirement for HSAF gene regulation was noted by us earlier (25). Clp is a c-di-GMP-responsive transcription factor that has been characterized in *Xanthomonas* species (33) but not yet in *Lysobacter*. It remains to be determined whether *L. enzymogenes* Clp activates HSAF gene expression directly and whether it responds to intracellular c-di-GMP levels.

Which c-di-GMP signaling systems are controlled by PilR and in turn affect HSAF gene expression remain unknown. Like many environmental proteobacteria, *L. enzymogenes* contains a large set of enzymes (26 enzymes) that are potentially involved in c-di-GMP synthesis and hydrolysis (14). Why elevated c-di-GMP levels are inhibitory for HSAF production also remains unknown. Consistent with the notion that c-di-GMP signaling plays an important role in HSAF production is our earlier observation that HSAF production was lower in the *rpfG* mutant (13). The RpfG protein is an RR containing an HD-GYP domain, which is predicted, based on its similarity to RpfG from *Xanthomonas* (34), to have c-di-GMP phosphodiesterase activity. Our work also contributes to the growing realization of the importance of c-di-GMP signaling pathways for the production of secondary metabolites in diverse bacteria. Earlier studies with *Streptomyces coelicolor* and *P. aeruginosa* identified the engagement of c-di-GMP pathways in the regulation of pigment and antibiotic synthesis (35).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The complete list of bacterial strains and plasmids used in this study is presented in Table 1. *L. enzymogenes* OH11 (6) was used as the wild-type strain. The deletion mutants in the RR genes were made in the OH11 background and designated Δ*Le#* (the number sign indicates the gene number). *Escherichia coli* strains DH5α and BL21(DE3), which were used for plasmid maintenance and protein overexpression, respectively, were routinely grown at 37°C in Luria broth (LB) supplemented with appropriate antibiotics (25 μg/ml gentamicin [Gm] or 100 μg/ml ampicillin [Ap]) and 100 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). *L. enzymogenes* was grown at 28°C in LB or TSB. When required, antibiotics were added at the final concentrations of 25 μg/ml kanamycin (Km) or 150 μg/ml Gm.

Bioinformatics analysis. The putative HKs and RRs in *L. enzymogenes* strain OH11 (9) were identified by using the Pfam 28.0 database (36).

Genetic methods. In-frame deletions in *L. enzymogenes* OH11 were generated via double-crossover homologous recombination, as described previously (37). The primers used are listed in Table 2. In brief, the flanking regions of each gene were amplified by PCR and cloned into the suicide vector pEX18Gm (Table 1). The deletion constructs were transformed into the wild-type strain OH11 or its derivatives by electroporation. The single-crossover recombinants were selected on LB plates supplemented with Km

TABLE 2 Primers used in this study

Primer	Sequence[a]	Purpose
In-frame deletion		
0041-F1	GGGGTACCGGCTTCCCGTTTCACCCTG (KpnI)	To amplify 920-bp upstream homologue arm of *Le0041*
0041-R1	CCCAAGCTTGATCCAGCGCAGTCCGTGA (HindIII)	
0041-F2	CCCAAGCTTCGGCGAAGGGGCGTTGAT (HindIII)	To amplify 646-bp downstream homologue arm of *Le0041*
0041-R2	GCTCTAGATGGAGCGTGTCGGGCTGGTC (XbaI)	
0371-F1	GGGGTACCTGCTGATGCTCGCCCACG (KpnI)	To amplify 500-bp upstream homologue arm of *Le0371*
0371-R1	CCCAAGCTTATGCCCGGCATCATCAGGT (HindIII)	
0371-F2	CCCAAGCTTGGGTTGATGCGCCGGAAGGA (HindIII)	To amplify 336-bp downstream homologue arm of *Le0371*
0371-R2	GCTCTAGAGGTGCTGAGCCTGTCCAACGAG (XbaI)	
0445-F1	GGGGTACCCGGCATTTCGTGCGTAGCG (KpnI)	To amplify 997-bp upstream homologue arm of *Le0445*
0445-R1	CCCAAGCTTCTCGGAAGCCACGGTCAAGG (HindIII)	
0445-F2	CCCAAGCTTTCAGGTCGAGCAGGAGCAGG (HindIII)	To amplify 1,132-bp downstream homologue arm of *Le0445*
0445-R2	GCTCTAGACGGCGAGCAAGCCGTTCTT (XbaI)	
0537-F1	CCCGGTACCAGCAGCAACAGCCAGCCGAT (KpnI)	To amplify 787-bp upstream homologue arm of *Le0537*
0537-R1	CCCTCTAGAACGGTCAGGGCGAAGGTCAT (XbaI)	
0537-F2	CCCTCTAGAATCACGAACACTCGAATCAT (XbaI)	To amplify 766-bp downstream homologue arm of *Le0537*
0537-R2	CCCAAGCTTTTTTGAAGAATGGAGACGCG (HindIII)	
0598-F1	CCCGGTACCCTGCCGGTCCTGCACGGTCT (KpnI)	To amplify 592-bp upstream homologue arm of *Le0598*
0598-R1	CCCTCTAGAGGCCTGGGTTACGTGGTCG (XbaI)	
0598-F2	CCCTCTAGAAGGTCGTAGGTGCCGTCGTG (XbaI)	To amplify 647-bp downstream homologue arm of *Le0598*
0598-R2	CCCAAGCTTTTGCCATCGTGTTGTCCCCT (HindIII)	
0752-F1	CGGAATTCGGCATAGCCGATCACCTCG (EcoRI)	To amplify 502-bp upstream homologue arm of *Le0752*
0752-R1	CCCAAGCTTTCGCGTCGTCTTCCACCAG (HindIII)	
0752-F2	CCCAAGCTTCAACACCATCGCGGTGTACG (HindIII)	To amplify 354-bp downstream homologue arm of *Le0752*
0752-R2	GCTCTAGACCGCCTTGAACAGCACCCA (XbaI)	
0760-F1	CGGGGTACCAGAGTCGGTCGTCGTGGGCG (KpnI)	To amplify 346-bp upstream homologue arm of *Le0760*
0760-R1	CCCAAGCTTCGAGGGCACGGTCAAGAATT (HindIII)	
0760-F2	CCCAAGCTTGGCATGCGGATGTCGCTGAGCACCA (HindIII)	To amplify 738-bp downstream homologue arm of *Le0760*
0760-R2	GCTCTAGATCACCGCGATGATGCTGAACC (XbaI)	
0872-F1	GGGGTACCCGTAGGCGTCGGAGATGGTC (KpnI)	To amplify 546-bp upstream homologue arm of *Le0872*
0872-R1	CCCAAGCTTGCGGCTGCGGCTGAAAGGCT (HindIII)	
0872-F2	CCCAAGCTTGGTTGGGGTTCTTCGTCGGC (HindIII)	To amplify 528-bp downstream homologue arm of *Le0872*
0872-R2	GCTCTAGACTTCCCGTTCGCTCCCGTAC (XbaI)	
0906-F1	GGGGTACCTGCGGACAAGGTGGTGGACT (KpnI)	To amplify 378-bp upstream homologue arm of *Le0906*
0906-R1	CCCAAGCTTCGTAGGTCTCGTCGTCGTCG (HindIII)	
0906-F2	CCCAAGCTTGGCGGCAACATCTCGGCGAC (HindIII)	To amplify 683-bp downstream homologue arm of *Le0906*
0906-R2	GCTCTAGAATTCGCTCCTGTTCGCCGCC (XbaI)	
0916-F1	CGGGGTACCGGTGCGTGGAAAGGGTCAGG (KpnI)	To amplify 932-bp upstream homologue arm of *Le0916*
0916-R1	CCCAAGCTTGTGCTCGGCATCAGCGTCAA (HindIII)	
0916-F2	CCCAAGCTTAGGTAGATGCGGGCTTCGC (HindIII)	To amplify 788-bp downstream homologue arm of *Le0916*
0916-R2	GCTCTAGACGTGTTCGGGTTCACCTTGC (XbaI)	
0979-F1	GGGGTACCTTGTTCCTGCCGCTGGTGTC (KpnI)	To amplify 386-bp upstream homologue arm of *Le0979*
0979-R1	CCCAAGCTTTTTCTCCAGCAACGCCAGCC (HindIII)	
0979-F2	CCCAAGCTTTGGGACGCAACACGCTCACG (HindIII)	To amplify 286-bp downstream homologue arm of *Le0979*
0979-R2	GCTCTAGAATTATGGCGGCGATGCGGGC (XbaI)	
1110-F1	CGGAATTCGAGAACAACCCGCTGCCGAG (EcoRI)	To amplify 873-bp upstream homologue arm of *Le1110*
1110-R1	CCCAAGCTTGTCCACCACCATCACCCGCG (HindIII)	
1110-F2	CCCAAGCTTCGAGGAGCGATTGCTGGTGA (HindIII)	To amplify 589-bp downstream homologue arm of *Le1110*
1110-R2	GCTCTAGACCCACAGCAGGAACACCAATC (XbaI)	
1120-F1	GGGGTACCGCAGGAGCAGGAATCGCCGC (KpnI)	To amplify 725-bp upstream homologue arm of *Le1120*
1120-R1	CCCAAGCTTTGTCGGGCAGTTCGTCGCGC (HindIII)	
1120-F2	CCCAAGCTTGCTACCGCCTCGCCGTGCCG (HindIII)	To amplify 856-bp downstream homologue arm of *Le1120*
1120-R2	GCTCTAGAGCGAGGTGCGGCGGATGCGG (XbaI)	
1130-F1	GGGGTACCCCAGCACCACGCACGGCACC (KpnI)	To amplify 728-bp upstream homologue arm of *Le1130*
1130-R1	CCCAAGCTTCGCAGCACCACCGAGGTCTG (HindIII)	
1130-F2	CCCAAGCTTAGACCGTGTGGGGACGAGGC (HindIII)	To amplify 406-bp downstream homologue arm of *Le1130*
1130-R2	GCTCTAGAGGCAGGCGCAGGAACAGGTG (XbaI)	
1234-F1	CCCGGTACCTGTAGCCCCAGCCGTAGAAC (KpnI)	To amplify 677-bp upstream homologue arm of *Le1234*
1234-R1	CCCTCTAGACGAAGCCCTGGTAACGCAGC (XbaI)	
1234-F2	CCCTCTAGACGGGCTACATGATCGAGGC (XbaI)	To amplify 749-bp downstream homologue arm of *Le1234*
1234-R2	CCCAAGCTTCGGTTCAACGATTCCACGG (HindIII)	
1263-F1	CCCGGTACCGACGAGCACGCCGAACAAGG (KpnI)	To amplify 675-bp upstream homologue arm of *Le1263*
1263-R1	CCCTCTAGACGAACGCCGAGGAAGTGCTG (XbaI)	
1263-F2	CCCTCTAGAGCAGGTCGGCTTGGTCTTCG (XbaI)	To amplify 594-bp downstream homologue arm of *Le1263*
1263-R2	CCCAAGCTTATCAGCGAGCAGCCCAGGCG (HindIII)	

(Continued on next page)

TABLE 2 (Continued)

Primer	Sequence[a]	Purpose
1423-F1	GGGGTACCGTGTCGGAGGAACGCAACCG (KpnI)	To amplify 828-bp upstream homologue arm of *Le1423*
1423-R1	CCCAAGCTTCGTCGGGCATCATCAGGTCG (HindIII)	
1423-F2	CCCAAGCTTCAAGTCGCACCTGGGCAACG (HindIII)	To amplify 516-bp downstream homologue arm of *Le1423*
1423-R2	GCTCTAGACACAGCAGGGAGCGGGAAAG (XbaI)	
1446-F1	GGGGTACCCAAGCCGCATTTCCTGTTCAA (KpnI)	To amplify 612-bp upstream homologue arm of *Le1446*
1446-R1	CCCAAGCTTCGCCAACGGTTCGTCGTCA (HindIII)	
1446-F2	CCCAAGCTTCGAGGAATCGCTCAAGTCGC (HindIII)	To amplify 1,130-bp downstream homologue arm of *Le1446*
1446-R2	GCTCTAGACTCATCGGCACCAGGGTCA (XbaI)	
1610-F1	CCCGGTACCTCGTTCCTGCGCTCGTGGTC (KpnI)	To amplify 676-bp upstream homologue arm of *Le1610*
1610-R1	CCCTCTAGACAGCCAGTTCGGGTTCGTCTTC (XbaI)	
1610-F2	CCCTCTAGACGGGGTCGGCTATCTGCTGC (XbaI)	To amplify 487-bp downstream homologue arm of *Le1610*
1610-R2	CCCAAGCTTCTTTCTCGGTGGTCAGGATC (HindIII)	
1647-F1	CCCGGTACCTAAAAAAAGTTCATCCGCCG (KpnI)	To amplify 535-bp upstream homologue arm of *Le1647*
1647-R1	CCCTCTAGACGCATACCGCCTCCGAAAGC (XbaI)	
1647-F2	CCCTCTAGAACTACCACTTCGACCCGCAG (XbaI)	To amplify 681-bp downstream homologue arm of *Le1647*
1647-R2	CCCAAGCTTCAGCATCAAGCCGAGGAAGC (HindIII)	
pilR-F1	CCCGGTACCTAGGAGTGATTGGTTGCTTC (KpnI)	To amplify 688-bp upstream homologue arm of *pilR*
pilR-R1	CCCTCTAGAGAGAACCGCTACAACAAGAC (XbaI)	
pilR-F2	CCCTCTAGAGTTTCAGCCATTACGCCCTC (XbaI)	To amplify 756-bp downstream homologue arm of *pilR*
pilR-R2	CCCAAGCTTGCCCACGAGATCCGCAATCC (HindIII)	
1910-F1	GGGGTACCATGGCAACGGAATCCTCAA (KpnI)	To amplify 901-bp upstream homologue arm of *Le1910*
1910-R1	CCCAAGCTTCATCACCAGATCGGGCAACT (HindIII)	
1910-F2	CCCAAGCTTTGCTGGATAAGTGAACCGATGA (HindIII)	To amplify 471-bp downstream homologue arm of *Le1910*
1910-R2	GCTCTAGAGACGAAATGGGCGTAGCG (XbaI)	
1921-F1	GGGGTACCCGGAACAACTGGAATCGCTC (KpnI)	To amplify 335-bp upstream homologue arm of *Le1921*
1921-R1	CCCAAGCTTGCGATGCGTTGGCGGATCAC (HindIII)	
1921-F2	CCCAAGCTTCCCTGCTGGAGATGCTGCCTAC (HindIII)	To amplify 1,039-bp downstream homologue arm of *Le1921*
1921-R2	GCTCTAGACGGGAAACGCCTGCAACA (XbaI)	
1936-F1	CGGAATTCAGGGGTGGTGTGTGATGGCC (EcoRI)	To amplify 249-bp upstream homologue arm of *Le1936*
1936-R1	GCTCTAGAGCTGTTCTCCGTCGCCAACC (XbaI)	
1936-F2	GCTCTAGAGCCACCTCTACAACCTGCGC (XbaI)	To amplify 226-bp downstream homologue arm of *Le1936*
1936-R2	CGAGCTCAGGAGGATGCCCAGCGTGAT (SacI)	
2134-F1	GGGGTACCCGCCAACACCTTCATCCCGC (KpnI)	To amplify 781-bp upstream homologue arm of *Le2134*
2134-R1	CCCAAGCTTACACCAGCGAAGAGAAGCCC (HindIII)	
2134-F2	CCCAAGCTTACGGGGTCGGCGGGTTGAGT (HindIII)	To amplify 470-bp downstream homologue arm of *Le2134*
2134-R2	GCTCTAGACTGCTCGATTACCGCCTGGG (XbaI)	
2333-F1	CGGAATTCCTTTGTCGGTGGTGGTGCTGAA (EcoRI)	To amplify 683-bp upstream homologue arm of *Le2333*
2333-R1	CCCAAGCTTCGATGTCGGGTTCCAGGTTCA (HindIII)	
2333-F2	CCCAAGCTTGCAACCGGATCGAGGCGTAT (HindIII)	To amplify 533-bp downstream homologue arm of *Le2333*
2333-R2	GCTCTAGAGGCGGAAGGTCGTAATGGAAGT (XbaI)	
2666-F1	CCCGGTACCCGCCCGCATCGCTTGATA (KpnI)	To amplify 899-bp upstream homologue arm of *Le2666*
2666-R1	CCCTCTAGATCCTGGTCGGCAAACTGC (XbaI)	
2666-F2	CCCTCTAGACTTGCGGATCTGCGGTTCGT (XbaI)	To amplify 704-bp downstream homologue arm of *Le2666*
2666-R2	CCCAAGCTTGAAACCATCCGCATCGAAGG (HindIII)	
2949-F1	GGGGTACCGGCGACGATGGGCTTGCT (KpnI)	To amplify 651-bp upstream homologue arm of *Le2949*
2949-R1	CCCAAGCTTCGCCAGCACCTGGCAGAAC (HindIII)	
2949-F2	CCCAAGCTTGTGGACCGGCGCACCTTG (HindIII)	To amplify 940-bp downstream homologue arm of *Le2949*
2949-R2	GCTCTAGAGAACGGGCGGACTTGATG (XbaI)	
3126-F1	GGGGTACCGGCTGGGTCGGGCTGGAATC (KpnI)	To amplify 329-bp upstream homologue arm of *Le3126*
3126-R1	CCCAAGCTTGTTCGTCGTCCTCGTCGTCG (HindIII)	
3126-F2	CCCAAGCTTTCAAGACCTTGGAGTGGGAACG (HindIII)	To amplify 416-bp downstream homologue arm of *Le3126*
3126-R2	GCTCTAGACTCGGTCGGTGTTCGTCCAT (XbaI)	
3199-F1	GGGGTACCACCACACCATCGCCCAGGAC (KpnI)	To amplify 178-bp upstream homologue arm of *Le3199*
3199-R1	CCCAAGCTTGGGAGTGGGGAAGGAGGGTT (HindIII)	
3199-F2	CCCAAGCTTGCTGCTGATGCTGGATGTCG (HindIII)	To amplify 465-bp downstream homologue arm of *Le3199*
3199-R2	GCTCTAGATGCGTTTCCTGGGTGTCTGT (XbaI)	
3200-F1	GGGGTACCGGAATGAACCACGCCACAGC (KpnI)	To amplify 557-bp upstream homologue arm of *Le3200*
3200-R1	CCCAAGCTTCAGTCCTTCCAGCAACCGCG (HindIII)	
3200-F2	CCCAAGCTTTATTCGCCCAGACCCAGACC (HindIII)	To amplify 299-bp downstream homologue arm of *Le3200*
3200-R2	GCTCTAGAGGTGGATGCGGTAGTGGTGC (XbaI)	
3343-F1	GGGGTACCGCCTGGACCGGATCGGGATT (KpnI)	To amplify 315-bp upstream homologue arm of *Le3343*
3343-R1	CCCAAGCTTAGGTGCTGCGGCTGTTCGTC (HindIII)	
3343-F2	CCCAAGCTTTGCTCAGGCGGACAAAAGG (HindIII)	To amplify 966-bp downstream homologue arm of *Le3343*
3343-R2	GCTCTAGAAGCAGACGAGGACAGCCCAT (XbaI)	
3450-F1	GGGGTACCGGCGTGTCCCTGCTCGGCAT (KpnI)	To amplify 360-bp upstream homologue arm of *Le3450*
3450-R1	CCCAAGCTTCGTCTTCTGCGGTGAGGGCC (HindIII)	

(Continued on next page)

TABLE 2 (Continued)

Primer	Sequence[a]	Purpose
3450-F2	CCCAAGCTTCCGTTCAGCGAGACCGACCT (HindIII)	To amplify 269-bp downstream homologue arm of *Le3450*
3450-R2	GCTCTAGACAAAACGCTCCGCCGCCACT (XbaI)	
3590-F1	GGGGTACCGGAATCCTGTGCGGTCGTCTTG (KpnI)	To amplify 280-bp upstream homologue arm of *Le3590*
3590-R1	GGGGTACCGGAATCCTGTGCGGTCGTCTTG (HindIII)	
3590-F2	CCCAAGCTTTCTGTCGCCGCAGCAGTTCC (HindIII)	To amplify 392-bp downstream homologue arm of *Le3590*
3590-R2	GCTCTAGACCGCTGTCCGCAGGTTTGTC (XbaI)	
3696-F1	CCCGGTACCATCCCTGCCCCCATCGCTAC (KpnI)	To amplify 678-bp upstream homologue arm of *Le3696*
3696-R1	CCCTCTAGAAGGATGTGGTCGCTGGGTTT (XbaI)	
3696-F2	CCCTCTAGAGCTACATCAAGACCGTGCGC (XbaI)	To amplify 605-bp downstream homologue arm of *Le3696*
3696-R2	CCCAAGCTTCGCACAGCAGCAGCAACGCC (HindIII)	
3816-F1	GGGGTACCTCTGGTCGGAAGTGCTCG (KpnI)	To amplify 596-bp upstream homologue arm of *Le3816*
3816-R1	CCCAAGCTTGGTGGACTGCTGAAATGGC (HindIII)	
3816-F2	CCCAAGCTTTGATCGGCTCGCTGACCAAC (HindIII)	To amplify 656-bp downstream homologue arm of *Le3816*
3816-R2	GCTCTAGACAACGCACTCATGCTGCTTCAC (XbaI)	
4011-F1	GGGGTACCCGGTGAACTGCCGCTACTGC (KpnI)	To amplify 1,098-bp upstream homologue arm of *Le4011*
4011-R1	CCCAAGCTTGCCTCCATCATCACCAGCACC (HindIII)	
4011-F2	CCCAAGCTTGCGGCTACATGGAAGACCTGA (HindIII)	To amplify 724-bp downstream homologue arm of *Le4011*
4011-R2	GCTCTAGAGCGATGATGAGCGGCAACC (XbaI)	
4034-F1	CCCGGTACCAGACCAGGAAATACAGCGGC (KpnI)	To amplify 755-bp upstream homologue arm of *Le4034*
4034-R1	CCCTCTAGAGCCAAATCCTCAGCCGCGAC (XbaI)	
4034-F2	CCCTCTAGAGCCGTGCTTGCCCAGGTAAT (XbaI)	To amplify 566-bp downstream homologue arm of *Le4034*
4034-R2	CCCAAGCTTGCGGCGGGCTGCAAAAAAAT (HindIII)	
4042-F1	CCCGGTACCGACAGGTTGCTCGGGCTCAG (KpnI)	To amplify 685-bp upstream homologue arm of *Le4042*
4042-R1	CCCTCTAGAATGGGCTATGTGCTGGAGAC (XbaI)	
4042-F2	CCCTCTAGAAGGTAGTCGGCGGTCTTGGC (XbaI)	To amplify 718-bp downstream homologue arm of *Le4042*
4042-R2	CCCAAGCTTGGATGCCGAAACCGAAGCCG (HindIII)	
4104-F1	GGGGTACCCAGGGCGATGTAGGCGTTGC (KpnI)	To amplify 851-bp upstream homologue arm of *Le4104*
4104-R1	CCCAAGCTTAAGGCTCGGCTGGTGGGGTC (HindIII)	
4104-F2	CCCAAGCTTAGGTGGCGGGCGAGACGATC (HindIII)	To amplify 269-bp downstream homologue arm of *Le4104*
4104-R2	GCTCTAGAGGGAAACCGCCGAGCCAATC (XbaI)	
4189-F1	CCCGGTACCCCAAGAACAGCCTCACAGCG (KpnI)	To amplify 1,511-bp upstream homologue arm of *Le4189*
4189-R1	CCCTCTAGACGCAGGGCAAAGGACACCAT (XbaI)	
4189-F2	CCCTCTAGATACCGCTTCTCCGCCTCGCT (XbaI)	To amplify 621-bp downstream homologue arm of *Le4189*
4189-R2	CCCAAGCTTCAGCAGCCACAGGTTTTCCG (HindIII)	
4215-F1	CCCGGTACCATCAGCACGAAGCCCCAGCG (KpnI)	To amplify 611-bp upstream homologue arm of *Le4215*
4215-R1	CCCTCTAGATGGGCTATTCGCTGGACAAC (XbaI)	
4215-F2	CCCTCTAGAATGGCGGATTCGTCTTCTAC (XbaI)	To amplify 1,065-bp downstream homologue arm of *Le4215*
4215-R2	CCCAAGCTTTCTATTCGGGCTGCTTCAAC (HindIII)	
4260-F1	GGGGTACCATGCCGACGACCAGGAACA (KpnI)	To amplify 662-bp upstream homologue arm of *Le4260*
4260-R1	CCCAAGCTTGAGGGGACGATCAAGAACCAC (HindIII)	
4260-F2	CCCAAGCTTCCTCCAGGCCGGACATCA (HindIII)	To amplify 857-bp downstream homologue arm of *Le4260*
4260-R2	GCTCTAGAGGCTCAACGCCGAACTGC (XbaI)	
4303-F1	GGGGTACCGCCGCACTTCCTCTACAACACC (KpnI)	To amplify 659-bp upstream homologue arm of *Le4303*
4303-R1	CCCAAGCTTGCGCAATGCCTCGACCAA (HindIII)	
4303-F2	CCCAAGCTTGCTGAGCGTGAGCCAGACCTT (HindIII)	To amplify 477-bp downstream homologue arm of *Le4303*
4303-R2	GCTCTAGAGCGATGCGTTCGGTGATGC (XbaI)	
4778-F1	GGGGTACCTCAACGAGGACACCGAGCGC (KpnI)	To amplify 540-bp upstream homologue arm of *Le4778*
4778-R1	CCCAAGCTTATCAGCACGCTCAACGGGCG (HindIII)	
4778-F2	CCCAAGCTTTCGGTGGAACTGGCGGTGGG (HindIII)	To amplify 619-bp downstream homologue arm of *Le4778*
4778-R2	GCTCTAGACACCCATCCCGACGCCTACG (XbaI)	
5176-F1	GGGGTACCCTCGGAAGAACTGGGCAAGG (KpnI)	To amplify 859-bp upstream homologue arm of *Le5176*
5176-R1	CCCAAGCTTGTGGTGTCGGCGGTGAAGTT (HindIII)	
5176-F2	CCCAAGCTTACCGCCTCAACACCATCCAG (HindIII)	To amplify 598-bp downstream homologue arm of *Le5176*
5176-R2	GCTCTAGAGTGATAGAACAGCAGCGGCG (XbaI)	
5230-F1	GGGGTACCGGGCGATGGAAGCAAGGGTG (KpnI)	To amplify 1,257-bp upstream homologue arm of *Le5230*
5230-R1	CCCAAGCTTGCCAGCAGGGCGAGAATGT (HindIII)	
5230-F2	CCCAAGCTTCCGGGGCTGATCAAGGCG (HindIII)	To amplify 1,070-bp downstream homologue arm of *Le5230*
5230-R2	GCTCTAGAGCCTGCCTCGGGCTTGTC (XbaI)	
pilS-F1	CCCGGTACCTAGGAGTGATTGGTTGCTTC (EcoRI)	To amplify 999-bp upstream homologue arm of *pilS*
pilS-R1	CCCTCTAGAGAGAACCGCTACAACAAGAC (HindIII)	
pilS-F2	CCCTCTAGAGTTTCAGCCATTACGCCCTC (HindIII)	To amplify 970-bp downstream homologue arm of *pilS*
pilS-R2	CCCAAGCTTGCCCACGAGATCCGCAATCC (XbaI)	
0445-F	TCCCACAACAGCCGACAGCC	To confirm mutant construction of Δ*Le0445*
0445-R	CCACCTTCACCCATCGTCCAAT	
0537-F	ATCGCCGGATTCCGTTATG	To confirm mutant construction of Δ*Le0537*
0537-R	GGTATCGGTGATCGTGAGCC	

(Continued on next page)

TABLE 2 (Continued)

Primer	Sequence[a]	Purpose
0598-F	GCACCAGCAGGAACAGCAGC	To confirm mutant construction of Δ*Le0598*
0598-R	GGCTTTGTAACCGTGCGTATCG	
0760-F	CGATGCGAAAGCGGAGATGG	To confirm mutant construction of Δ*Le0760*
0760-R	CGAACTGCTCGGCGACATCC	
0906-F	GCAACCACAGGCATGGACACTT	To confirm mutant construction of Δ*Le0906*
0906-R	CACCTGATGCTGATCGGATTGC	
0916-F	CGATGTCCGCTTGCGTATCAG	To confirm mutant construction of Δ*Le0916*
0916-R	CAACCAACAGTTCCCGCCCTAT	
1120-F	TGCGGGAATGATCGAAACGG	To confirm mutant construction of Δ*Le1120*
1120-R	CCGAACAGGCCGAGCAGGAT	
1130-F	TGAAGCGATTCGGGTCCAGC	To confirm mutant construction of Δ*Le1130*
1130-R	TGAGGTACAACCGCACCAGCA	
1234-F	AGCCGTAGAACTTGCCCGACAC	To confirm mutant construction of Δ*Le1234*
1234-R	TGGACACGCGGTAGAACACCC	
1446-F	CGTCCAAGACCGACTCCAGC	To confirm mutant construction of Δ*Le1446*
1446-R	TCACAGGTGCTTCAAGGTCTCG	
1610-F	AGATGCTGGGCGAGCGTTTCC	To confirm mutant construction of Δ*Le1610*
1610-R	CGTCGCGGATCACGTACCACA	
1647-F	CGTCGCACAAGCACAAGAAGC	To confirm mutant construction of Δ*Le1647*
1647-R	CGATGCCGAGCAGCACGAA	
pilR-F	CGGAGGCGATACTGGGAATG	To confirm mutant construction of Δ*pilR*
pilR-R	GGAGGGCGTAATGGCTGAAAC	
1921-F	CCGGGACCATTTCCATGTCG	To confirm mutant construction of Δ*Le1921*
1921-R	AAGTGCTTGCGGGCGTTGC	
2333-F	ACGCCTGAGCCTGCTGGTCT	To confirm mutant construction of Δ*Le2333*
2333-R	GGTTCGGATCGGGAAGGAGAA	
3200-F	GGACCCCGCAGTGAGGATAGG	To confirm mutant construction of Δ*Le3200*
3200-R	CGCTGGGAGTGGGGAAGGAG	
4104-F	GGTCCGCAGCATGGAAGCA	To confirm mutant construction of Δ*Le4104*
4104-R	CGAGCCAATCGGCGCTGTAC	
4215-F	ATCACCGTGTCGTCGGGATTG	To confirm mutant construction of Δ*Le4215*
4215-R	GTTTCCCTTCATTTCCCTGCTCC	
4778-F	CAGAACCCACCCTCGGAAAGC	To confirm mutant construction of Δ*Le4778*
4778-R	CGACGTGTTGAGCCAGGAAGG	
Construction of complementary plasmids		
pilR-cpF	CCCAAGCTTCGCACGGCAAGCAGAAAA (HindIII)	To amplify 1,540-bp fragment containing intact sequence of *pilR*
pilR-cpR	GCTCTAGAAGGGCGGGAACGACCCTGT (XbaI)	
Protein expression		
pilR-pET-F	GGAATTCCATATGGCTGAAACCCGTAGTGCATT (NdeI)	To amplify fragment of intact *pilR* sequence
pilR-pET-R	CCCAAGCTTGTCGATCCCGAGCTTCTTCA (HindIII)	
Biotin-labeled probe for EMSA analysis		
pilA-biotin-F	5′-Biotin-CGCCACGTAGCCGCCCGCCG-3′	To amplify 541-bp biotin probe of promoter region of *pilA*
pilA-biotin-R	5′-GGTGTATCCCCTAGGAGTGA-3′	
pilA-cold-F	5′-CGCCACGTAGCCGCCCGCCG-3′	To amplify 541-bp cold probe of promoter region of *pilA*
pilA-cold-R	5′-GGTGTATCCCCTAGGAGTGA-3′	
lafB-biotin-F	5′-Biotin-AATGATCCGCGTCGCAGGAT-3′	To amplify 491-bp biotin probe of promoter region of *lafB*
lafB-biotin-R	5′-CAGCAGCGGGTGGGCGCAGT-3′	
lafB-cold-F	5′-AATGATCCGCGTCGCAGGAT-3′	To amplify 491-bp cold probe of promoter region of *lafB*
lafB-cold-R	5′-CAGCAGCGGGTGGGCGCAGT-3′	
qRT-PCR analysis[b]		
pilA-qRT-F	TACAACTTCACCGCCAACAG	
pilA-qRT-R	TCAGGGTGAACTTGATGTCG	
lafB-qRT-F	ACTATTTGTTGGGCGACGAC	
lafB-qRT-F	GTAACCGAACAGGGTGCAA	
16S-qRT-F	ACGGTCGCAAGACTGAAACT	
16S-qRT-F	AAGGCACCAATCCATCTCTG	

[a]Underlined nucleotide sequences are restriction sites, and the restriction enzymes are indicated at the end of primers.
[b]From reference 25.

and Gm. The recombinants were then cultured for 6 h in liquid LB without antibiotics and subsequently were grown on LB plates containing 10% (wt/vol) sucrose and Km, for double-crossover enrichment. The sucrose-resistant, Km-resistant, Gm-sensitive colonies representing double-crossover recombinants were picked. In-frame gene deletions were verified by PCR using appropriate primers (Table 2).

Complementation constructs for each mutant were generated as described previously (12). In brief, the DNA fragments containing full-length genes along with their upstream promoter regions were amplified by PCR and cloned into the broad-host-range vectors pBBR1-MCS5 and pUFR047 (Table 1).

Twitching motility assays. *L. enzymogenes* twitching motility was assayed as described previously (14, 25). Briefly, bacteria were inoculated at the edge of a sterilized coverslip containing a thin layer of 0.05× tryptic soy agar (TSA). After 24 h of incubation, the margin of the bacterial culture on the microscope slide was observed. Cell clusters growing away from the main colony represented motile cells (14). Three slides for each treatment were used, and each experiment was performed three times.

HSAF extraction and quantification. HSAF was extracted from 25-ml *L. enzymogenes* cultures grown for 48 h at 28°C in 0.1× TSB, with shaking at 200 rpm. HSAF was detected by HPLC, as described previously (12), and quantified per unit of optical density at 600 nm (OD_{600}), as described previously (25). Three biological replicates were used, and each was assayed in three technical replicates.

RNA extraction and qRT-PCR. Cells were grown in 0.1× TSB or 0.05× TSB and collected at an OD_{600} of 1.0. RNA was extracted using a bacterial RNA kit (Omega, China), according to the manufacturer's protocol. Real-time qRT-PCR was performed using the 16S rRNA gene as an internal control, as described previously (12, 32). Primers for qRT-PCR are listed in Table 2. The primers used for measuring *pilA* and *lafB* mRNA were reported previously (12, 25).

Protein purification and EMSA. The full-length *pilR* coding sequence was amplified and cloned into the expression vector pET30a(+) to generate a *pilR*-His_6 fusion [plasmid PilR-pET30(a)]. *E. coli* BL21(DE3) [PilR-pET30(a)] was grown at 37°C, with shaking at 200 rpm, until the OD_{600} was 0.6. *pilR* expression was induced with isopropyl β-D-1-thiogalactopyranoside (0.5 mM final concentration), followed by incubation at 37°C for 6 h. The cells were collected by centrifugation, resuspended in 25 ml of lysis buffer, i.e., phosphate-buffered saline (PBS) containing 10 mM phenylmethylsulfonyl fluoride (PMSF) (a protease inhibitor), and lysed by sonication (Branson 250 digital sonifier). Following centrifugation at 13,000 rpm for 30 min at 4°C, the soluble extract was collected and mixed with preequilibrated Ni^{2+} resin (GE Health) for 1 h at 4°C. A column containing resin with bound PilR-His_6 was washed extensively with resuspension buffer, i.e., 50 mM PBS containing 30 mM imidazole and 300 mM NaCl. The PilR-His_6 protein was eluted with 250 mM imidazole. Finally, the protein eluent was transferred into an ultrafiltration device and concentrated by centrifugation at 3,000 × *g*.

An EMSA was performed as follows. The fragments containing promoter regions of *pilA* or *lafB* were amplified by PCR using biotin-5′-end-labeled primers (Table 2). The biotin-end-labeled target DNA and protein extract were incubated in binding reactions for the test system for 20 min at room temperature, according to the protocols of the LightShift chemiluminescent EMSA kit (Thermo). The binding reaction mixtures were then loaded onto a polyacrylamide (8%) gel, electrophoresed in 0.5× Tris-borate-EDTA (TBE) buffer, transferred to a nylon membrane, and cross-linked. Finally, the biotinylated DNA fragments were detected by chemiluminescence with a VersaDoc imaging system (Bio-Rad).

c-di-GMP extraction and quantification. Cultures were grown in 0.1× TSB at 28°C until the cell density reached an OD_{600} of 1.5. Cells from 2-ml cultures were harvested for protein quantification by the bicinchoninic acid (BCA) assay (TransGen). Cells from 8 ml of culture were used for c-di-GMP extraction with 0.6 M $HClO_4$ and 2.5 M K_2CO_3, as described previously (29, 38). The samples were analyzed by LC-MS, as described previously (29, 38, 39).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.03397-16.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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G.Q. and F.L. conceived the project, M.G., G.Q, and F.L. designed the experiments, Y.C., J.X., Z.S., and G.X. carried out the experiments, Y.C., M.G., G.Q., and F.L. analyzed the data, G.Q. wrote the manuscript draft, and M.G. and F.L. revised the manuscript.

REFERENCES

1. Christensen P, Cook FD. 1978. *Lysobacter*, a new genus of nonhiting, gliding bacteria with a high base ratio. Int J Syst Bacteriol 28:367–393. https://doi.org/10.1099/00207713-28-3-367.
2. Hayward AC, Fegan N, Fegan M, Stirling GR. 2010. *Stenotrophomonas* and *Lysobacter*: ubiquitous plant-associated *gamma*-proteobacteria of developing significance in applied microbiology. J Appl Microbiol 108: 756–770. https://doi.org/10.1111/j.1365-2672.2009.04471.x.
3. de Bruijn I, Cheng X, de Jager V, Exposito RG, Watrous J, Patel N, Postma J, Dorrestein PC, Kobayashi D, Raaijmakers JM. 2015. Comparative genomics and metabolic profiling of the genus *Lysobacter*. BMC Genomics 16:991. https://doi.org/10.1186/s12864-015-2191-z.
4. Zhang Z, Yuen GY. 1999. Biological control of *Bipolaris sorokiniana* on tall fescue by *Stenotrophomonas maltophilia* strain C3. Phytopathology 89: 817–822. https://doi.org/10.1094/PHYTO.1999.89.9.817.
5. Jochum CC, Osborne LE, Yuen GY. 2006. Fusarium head blight biological control with *Lysobacter enzymogenes*. Bio Control 39:336–344. https://doi.org/10.1016/j.biocontrol.2006.05.004.
6. Qian GL, Hu BS, Jiang YH, Liu FQ. 2009. Identification and characterization of *Lysobacter enzymogenes* as a biological control agent against some fungal pathogens. Agric Sci China 8:68–75. https://doi.org/10.1016/S1671-2927(09)60010-9.
7. Li S, Jochum CC, Yu F, Zaleta-Rivera K, Du L, Harris SD, Yuen GY. 2008. An antibiotic complex from *Lysobacter enzymogenes* strain C3: antimicrobial activity and role in plant disease control. Phytopathology 98:695–701. https://doi.org/10.1094/PHYTO-98-6-0695.
8. Yu F, Zaleta-Rivera K, Zhu X, Huffman J, Millet JC, Harris SD, Yuen G, Li XC, Du L. 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. https://doi.org/10.1128/AAC.00931-06.
9. Lou L, Qian G, Xie Y, Hang J, Chen H, Zaleta-Rivera K, Li Y, Shen Y, Dussault PH, Liu F, Du L. 2011. Biosynthesis of HSAF, a tetramic acid-containing macrolactam from *Lysobacter enzymogenes*. J Am Chem Soc 133:643–645. https://doi.org/10.1021/ja105732c.
10. Li Y, Chen H, Ding Y, Xie Y, Wang H, Cerny RL, Shen Y, Du L. 2014. Iterative assembly of two separate polyketide chains by the same single-module bacterial polyketide synthase in the biosynthesis of HSAF. Angew Chem Int Ed Engl 53:7524–7530. https://doi.org/10.1002/anie.201403500.
11. Li S, Du L, Yuen G, Harris SD. 2006. Distinct ceramide synthases regulate polarized growth in the filamentous fungus *Aspergillus nidulans*. Mol Biol Cell 17:1218–1227.
12. Qian G, Wang Y, Liu Y, Xu F, He YW, Du L, Venturi V, Fan J, Hu B, Liu F. 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. https://doi.org/10.1128/AEM.01841-13.
13. Han Y, Wang Y, Tombosa S, Wright S, Huffman J, Yuen G, Qian G, Liu F, Shen Y, Du L. 2015. Identification of a small molecule signaling factor that regulates the biosynthesis of the antifungal polycyclic tetramate macrolactam HSAF in *Lysobacter enzymogenes*. Appl Microbiol Biotechnol 99:801–811. https://doi.org/10.1007/s00253-014-6120-x.
14. Zhou X, Qian G, Chen Y, Du L, Liu F, Yuen GY. 2015. PilG is involved in the regulation of twitching motility and antifungal antibiotic biosynthesis in the biological control agent *Lysobacter enzymogenes*. Phytopathology 105:1318–1324. https://doi.org/10.1094/PHYTO-12-14-0361-R.
15. Romling U, Galperin MY, Gomelsky M. 2013. Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. Microbiol Mol Biol Rev 77:1–52. https://doi.org/10.1128/MMBR.00043-12.
16. Hengge R. 2009. Principles of c-di-GMP signalling in bacteria. Nat Rev Microbiol 7:263–273. https://doi.org/10.1038/nrmicro2109.
17. Ishimoto KS, Lory S. 1992. Identification of *pilR*, which encodes a transcriptional activator of the *Pseudomonas aeruginosa* pilin gene. J Bacteriol 174:3514–3521. https://doi.org/10.1128/jb.174.11.3514-3521.1992.
18. Hobbs M, Collie ES, Free PD, Livingston SP, Mattick JS. 1993. PilS and PilR, a two-component transcriptional regulatory system controlling expression of type 4 fimbriae in *Pseudomonas aeruginosa*. Mol Microbiol 7:669–682. https://doi.org/10.1111/j.1365-2958.1993.tb01158.x.
19. Boyd JM, Koga T, Lory S. 1994. Identification and characterization of PilS, an essential regulator of pilin expression in *Pseudomonas aeruginosa*. Mol Gen Genet 243:565–574. https://doi.org/10.1007/BF00284205.
20. Wu SS, Kaiser D. 1997. Regulation of expression of the *pilA* gene in *Myxococcus xanthus*. J Bacteriol 179:7748–7758. https://doi.org/10.1128/jb.179.24.7748-7758.1997.
21. Kehl-Fie TE, Porsch EA, Miller SE, St Geme JW, III. 2009. Expression of *Kingella kingae* type IV pili is regulated by σ^{54}, PilS, and PilR. J Bacteriol 191:4976–4986. https://doi.org/10.1128/JB.00123-09.
22. Dunger G, Guzzo CR, Andrade MO, Jones JB, Farah CS. 2014. *Xanthomonas citri* subsp. *citri* type IV pilus is required for twitching motility, biofilm development, and adherence. Mol Plant Microbe Interact 27:1132–1147. https://doi.org/10.1094/MPMI-06-14-0184-R.
23. Dunger G, Llontop E, Guzzo CR, Farah CS. 2016. The *Xanthomonas* type IV pilus. Curr Opin Microbiol 30:88–97. https://doi.org/10.1016/j.mib.2016.01.007.
24. Jin S, Ishimoto KS, Lory S. 1994. PilR, a transcriptional regulator of piliation in *Pseudomonas aeruginosa*, binds to a *cis*-acting sequence upstream of the pilin gene promoter. Mol Microbiol 14:1049–1057. https://doi.org/10.1111/j.1365-2958.1994.tb01338.x.
25. Wang Y, Zhao Y, Zhang J, Zhao Y, Shen Y, Su Z, Xu G, Du L, Huffman JM, Venturi V, Qian G, Liu F. 2014. Transcriptomic analysis reveals new regulatory roles of Clp signaling in secondary metabolite biosynthesis and surface motility in *Lysobacter enzymogenes* OH11. Appl Microbiol Biotechnol 98:9009–9020. https://doi.org/10.1007/s00253-014-6072-1.
26. Chin KH, Lee YC, Tu ZL, Chen CH, Tseng YH, Yang JM, Ryan RP, McCarthy Y, Dow JM, Wang AH, Chou SH. 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. https://doi.org/10.1016/j.jmb.2009.11.076.
27. Ryjenkov DA, Tarutina M, Moskvin OV, Gomelsky M. 2005. Cyclic diguanylate is a ubiquitous signaling molecule in bacteria: insights into biochemistry of the GGDEF protein domain. J Bacteriol 187:1792–1798. https://doi.org/10.1128/JB.187.5.1792-1798.2005.
28. Schmidt AJ, Ryjenkov DA, Gomelsky M. 2005. The ubiquitous protein domain EAL is a c-di-GMP-specific phosphodiesterase: enzymatically active and inactive EAL domains. J Bacteriol 187:4774–4781. https://doi.org/10.1128/JB.187.14.4774-4781.2005.
29. Hickman JW, Harwood CS. 2008. Identification of FleQ from *Pseudomonas aeruginosa* as a c-di-GMP-responsive transcription factor. Mol Microbiol 69:376–389. https://doi.org/10.1111/j.1365-2958.2008.06281.x.
30. Matsuyama BY, Krasteva PV, Baraquet C, Harwood CS, Sondermann H, Navarro MV. 2016. Mechanistic insights into c-di-GMP-dependent control of the biofilm regulator FleQ from *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 113:E209–E218. https://doi.org/10.1073/pnas.1523148113.
31. Yaryura PM, Conforte VP, Malamud F, Roeschlin R, de Pino V, Castagnaro AP, McCarthy Y, Dow JM, Marano MR, Vojnov AA. 2015. XbmR, a new transcription factor involved in the regulation of chemotaxis, biofilm formation and virulence in *Xanthomonas citri* subsp. *citri*. Environ Microbiol 17:4164–4176. https://doi.org/10.1111/1462-2920.12684.
32. Qian G, Xu F, Venturi V, Du L, Liu F. 2014. Roles of a solo LuxR in the biological control agent *Lysobacter enzymogenes* strain OH11. Phytopathology 104:224–231. https://doi.org/10.1094/PHYTO-07-13-0188-R.

33. Tao F, He YW, Wu DH, Swarup S, Zhang LH. 2010. The cyclic nucleotide monophosphate domain of *Xanthomonas campestris* global regulator Clp defines a new class of cyclic di-GMP effectors. J Bacteriol 192:1020–1029. https://doi.org/10.1128/JB.01253-09.
34. Slater H, Alvarez-Morales A, Barber CE, Daniels MJ, Dow JM. 2000. A two-component system involving an HD-GYP domain protein links cell-cell signalling to pathogenicity gene expression in *Xanthomonas campestris*. Mol Microbiol 38:986–1003.
35. Liang ZX. 2015. The expanding roles of c-di-GMP in the biosynthesis of exopolysaccharides and secondary metabolites. Nat Prod Rep 32:663–683. https://doi.org/10.1039/C4NP00086B.
36. Finn RD, Coggill P, Eberhardt RY, Eddy SR, Mistry J, Mitchell AL, Potter SC, Punta M, Qureshi M, Sangrador-Vegas A, Salazar GA, Tate J, Bateman A. 2016. The Pfam protein families database: towards a more sustainable future. Nucleic Acids Res 44:D279–D285. https://doi.org/10.1093/nar/gkv1344.
37. Qian G, Wang Y, Qian D, Fan J, Hu B, Liu F. 2012. Selection of available suicide vectors for gene mutagenesis using *chiA* (a chitinase encoding gene) as a new reporter and primary functional analysis of *chiA* in *Lysobacter enzymogenes* strain OH11. World J Microbiol Biotechnol 28:549–557. https://doi.org/10.1007/s11274-011-0846-8.
38. Zhu B, Liu C, Liu S, Cong H, Chen Y, Gu L, Ma LZ. 2016. Membrane association of SadC enhances its diguanylate cyclase activity to control exopolysaccharides synthesis and biofilm formation in *Pseudomonas aeruginosa*. Environ Microbiol 18:3440–3452. https://doi.org/10.1111/1462-2920.13263.
39. Thormann KM, Duttler S, Saville RM, Hyodo M, Shukla S, Hayakawa Y, Spormann AM. 2006. Control of formation and cellular detachment from *Shewanella oneidensis* MR-1 biofilms by cyclic di-GMP. J Bacteriol 188:2681–2691. https://doi.org/10.1128/JB.188.7.2681-2691.2006.
40. Ortet P, Whitworth DE, Santaella C, Achouak W, Barakat M. 2015. P2CS: updates of the prokaryotic two-component systems database. Nucleic Acids Res 43:D536–D541. https://doi.org/10.1093/nar/gku968.
41. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene 212:77–86. https://doi.org/10.1016/S0378-1119(98)00130-9.
42. Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM, II, Peterson KM. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166:175–176. https://doi.org/10.1016/0378-1119(95)00584-1.
43. De Feyter R, Yang Y, Gabriel DW. 1993. Gene-for-genes interactions between cotton *R* genes and *Xanthomonas campestris* pv. *malvacearum avr* genes. Mol Plant Microbe Interact 6:225–237. https://doi.org/10.1094/MPMI-6-225.