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GENETICS AND MOLECULAR BIOLOGY

Interspecies and Intraspecies Signals Synergistically Regulate *Lysobacter enzymogenes* Twitching Motility

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ABSTRACT The twitching motility of bacteria is closely related to environmental adaptability and pathogenic behaviors. *Lysobacter* is a good genus in which to study twitching motility because of the complex social activities and distinct movement patterns of its members. Regardless, the mechanism that induces twitching motility is largely unknown. In this study, we found that the interspecies signal indole caused *Lysobacter* to have irregular, random twitching motility with significantly enhanced speed. Deletion of *qseC* or *qseB* from the two-component system for indole signaling perception resulted in the disappearance of rapid, random movements and significantly decreased twitching activity. Indole-induced, rapid, random twitching was achieved through upregulation of expression of gene cluster *pilE1-pilY*$_1$*1-pilX1-pilW1-pilV1-fimT1*. In addition, under conditions of extremely low bacterial density, individual *Lysobacter* cells grew and divided in a stable manner *in situ* without any movement. The intraspecies quorum-sensing signaling factor 13-methyltetradecanoic acid, designated *L. enzymogenes* diffusible signaling factor (*Le*DSF), was essential for *Lysobacter* to produce twitching motility through indirect regulation of gene clusters *pilM-pilN-pilO-pilP-pilQ* and *pilS1-pilR-pilA-pilB-pilC*. These results demonstrate that the motility of *Lysobacter* is induced and regulated by indole and *Le*DSF, which reveals a novel theory for future studies of the mechanisms of bacterial twitching activities.

IMPORTANCE The mechanism underlying bacterial twitching motility is an important research area because it is closely related to social and pathogenic behaviors. The mechanism mediating cell-to-cell perception of twitching motility is largely unknown. Using *Lysobacter* as a model, we found in this study that the interspecies signal indole caused *Lysobacter* to exhibit irregular, random twitching motility via activation of gene cluster *pilE1-pilY*$_1$*1-pilX1-pilW1-pilV1-fimT1*. In addition, population-dependent behavior induced by 13-methyltetradecanoic acid, a quorum-sensing signaling molecule designated *Le*DSF, was involved in twitching motility by indirectly regulating gene clusters *pilM-pilN-pilO-pilP-pilQ* and *pilS1-pilR-pilA-pilB-pilC*. The results demonstrate that the twitching motility of *Lysobacter* is regulated by these two signaling molecules, offering novel clues for exploring the mechanisms of twitching motility and population-dependent behaviors of bacteria.

KEYWORDS interspecies and intraspecies signal indole, interspecies and intraspecies signal *Le*DSF, *Lysobacter enzymogenes*, quorum-sensing, twitching motility

The genus *Lysobacter* comprises a group of ubiquitous environmental bacteria that are emerging as a new source of bioactive natural products (1, 2). *Lysobacter* species exhibit two characteristic features among environmental Gram-negative bacteria: the

Citation Feng T, Han Y, Li B, Li Z, Yu Y, Sun Q, Li X, Du L, Zhang X-H, Wang Y. 2019. Interspecies and intraspecies signals synergistically regulate *Lysobacter enzymogenes* twitching motility. Appl Environ Microbiol 85:e01742-19. https://doi.org/10.1128/AEM.01742-19.

Editor Harold L. Drake, University of Bayreuth

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Received 1 August 2019
Accepted 18 September 2019
Accepted manuscript posted online 20 September 2019
Published 14 November 2019

first is that they are prolific producers of lytic enzymes and antibiotics, and the second is that they exhibit population-dependent behavior, such as twitching motility and quorum-sensing activity. The first of those *Lysobacter* features has been well studied by several research groups; indeed, multiple species of the genus are known to produce promising antibiotics. For example, lysobactin from *Lysobacter* sp. ATCC 53042 is a potent cyclic depsipeptide that blocks cell wall biosynthesis in bacteria (3–5), and the WAP-8294A family of *Lysobacter enzymogenes* OH11 and *Lysobacter* sp. WAP-8294 exhibits potent activities against the Gram-positive pathogen methicillin-resistant *Staphylococcus aureus* (MRSA) (6–8). In addition to the WAP-8294A family, *L. enzymogenes* produces another promising antifungus antibiotic, heat-stable antifungal factor (HSAF), as well as its analogs, which are a family of polycyclic tetramate macrolactams with distinct modes of action and biosynthesis patterns (9–11). Moreover, *Lysobacter antibioticus* OH13 produces the broad-spectrum antibiotic phenazine (12). In contrast to the signaling pathways that control the biosynthesis of natural products, those that control twitching motility and related population-dependent activities in *Lysobacter* remain largely unknown.

Twitching motility is conferred by the type IV pilus (Tfp) in many bacteria that exhibit social and pathogenic activities (13–16). Tfp production and assembly are regulated by diverse transcriptional mechanisms. For instance, the Tfp-dependent motility of *Myxobacteria* is coregulated via distinct regulating pathways by the PilSR and PilS2R2 two-component systems (17). PilB and PilT play are motors of pilus extension and retraction, respectively, in *Myxococcus xanthus* (18). Furthermore, traction force microscopy was recently employed to show that the force generated during *Myxobacteria* twitching motility was higher when the cells were in a group than when they were individually isolated (19). PilS-PilR, which is essential for activating the major pilin-encoding gene *pilA*, has been recognized as an important regulatory pathway for Tfp formation in *Pseudomonas aeruginosa* (20, 21). In *Lysobacter*, 14 genes responsible for twitching motility have been identified, including *pilA*, *pilM*, *pilN*, *pilO*, *pilP*, *pilQ*, *pilR*, *pilG* and *clp* (22–24). Among the genes regulating twitching motility, *clp*, is a global regulator that can control the Tfp-dependent twitching motility of *L. enzymogenes* OH11 by directly binding to the promoter regions of *pilA* and *pilMNOPQ* (25).

Endogenous and xenogenous signaling play essential roles in multiple bacterial physiological and biochemical behaviors (26–30). In one study assessing *Lysobacter* signaling, a group of diffusible signaling factors (*Le*DSFs) in *L. enzymogenes* was identified (31); among them, 13-methyltetradecanoic acid was the major compound and was named *L. enzymogenes* DSF (*Le*DSF) in ensuing studies. DSF signaling is mediated by the two-component regulatory system RpfC/RpfG, which is responsible for sensing DSFs and for conveying the signal for subsequent gene expression (32). Although the downstream signal transduction pathway in *L. enzymogenes* is still unclear, evidence suggests that activated RpfG hydrolyzes cyclic di-GMP of Clp-c-di-GMP, a ubiquitous second messenger that controls numerous cellular processes by regulating intracellular protein effectors and riboswitches. This process leads to release of Clp, which is related to the cAMP receptor protein Crp, and activation of downstream genes (31, 33).

Indole is another type of ubiquitous interkingdom signaling molecule (34, 35). A recently study of ours reported that indole is able to reverse the intrinsic antibiotic resistance of *Lysobacter* through the two-component regulatory system QseC/QseB. Site-specific mutations in the *qseC* gene also resulted in antibiotic susceptibility (36). Besides, this antibiotic resistance reversal was facilitated by a novel BtuD-associated importer which can transfer both vitamin B12 and antibiotics (37). So we wanted to know whether other characteristics of *L. enzymogenes* are also related to indole. At the same time, some studies have reported that indole can affect the motility of some other bacteria, such as the indole-decreased motility of *Escherichia coli* strains and of *ymgA*, *ymgB* and *ymgC* mutants by 35% to 65% (38); also, exogenous indole can significantly reduce the number of flagella and inhibit the motility of *Salmonella enterica* serovar strains (39). However, with regard to twitching activity, it is largely unknown how

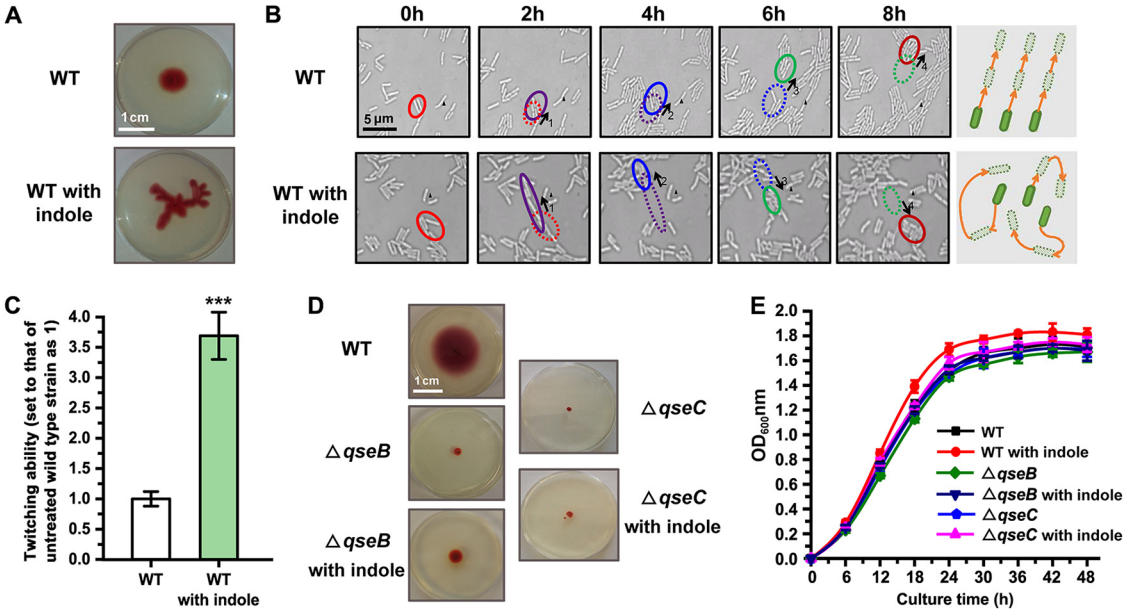

FIG 1 Indole regulates the twitching motility of *Lysobacter enzymogenes*. (A) A comparison of the twitching motility patterns of the untreated wild-type (WT) strain and the wild-type strain treated with 0.5 mM indole in motility test medium for 48 h. (B) Dynamic imaging assay under the microscope to detect the twitching motility patterns of the wild-type strain left untreated and/or treated with 0.5 mM indole. The red, purple, blue, green, and dark red circles represent the positions of the bacteria at 0, 2, 4, 6, and 8 h, respectively. 1, motion process at 0 to 2 h; 2, motion process at 2 to 4 h; 3, motion process at 4 to 6 h; 4, motion process at 6 to 8 h. The solid black triangle indicates a fixed position reference. The magnification setting of the microscope was ×640. (C) The speeds of twitching motility of the untreated wild-type strain and the wild-type strain treated with indole were compared. In this experiment, we set the value corresponding to the twitching speed of untreated bacteria to 1. (D) Analysis of twitching ability of the wild-type strain and Δ*qseC/B* mutants in the presence or absence of 0.5 mM indole. To show the results clearly, the chosen cultivation time was 72 h. The results are representative of biological duplicates. (E) Growth of the wild-type strain and Δ*qseC/B* mutants. The results are representative of biological duplicates. The error bars represent the standard deviations of results from at least three replicates. OD_{600}nm, optical density at 600 nm. A *t* test of unpaired unequal variances was performed for testing differences between groups. For statistical analysis, ***, **, and * indicate $P < 0.001$, $P < 0.01$, and $P < 0.05$, respectively.

xenogenous small molecules are recognized and transmitted by *Lysobacter* to regulate twitching activity and the Tfp system. In this study, we found that the interspecies signal indole caused *Lysobacter* to produce irregular random twitching motility by regulating the gene cluster *pilE1-pilY*$_1$*1-pilX1-pilW1-pilV1-fimT1*. In addition, the intraspecies quorum-sensing signaling factor 13-methyltetradecanoic acid, designated *Le*DSF, was also essential for *Lysobacter* to produce twitching motility through indirect regulation of gene clusters *pilM-pilN-pilO-pilP-pilQ* and *pilS1-pilR-pilA-pilB-pilC*. The synergistic regulation pattern opens a novel approach for studying the mechanism of bacterial twitching activity in the future.

RESULTS

Indole regulates the twitching motility of *Lysobacter enzymogenes*. The twitching motility of bacteria is involved in a variety of physiological and sociological behaviors. *L. enzymogenes*, a type of ubiquitous bacterium that possesses multiple social activities, displays obvious twitching motility and can spread uniformly on semisolid medium without directional selectivity. In this study, we showed that wild-type *L. enzymogenes* exhibited significantly increased twitching motility when treated with the interspecies signaling molecule indole. Interestingly, the motility pattern of the treated strain became apparently irregular compared with that of the untreated wild-type strain (Fig. 1A). To examine the reason for this phenomenon, we carried out a dynamic imaging assay, the results of which indicated that the twitching motility direction of untreated *L. enzymogenes* was uniform. As shown in Fig. 1B and Movies S1 and S2 in the supplemental material, a group of bacterial cells moved in the same direction at similar speeds, though a few cells were found to be irregularly rotating. However, in the presence of indole, regular motility was replaced by irregular movements with frequent

reorientation. The average speed of the twitching motility of *L. enzymogenes* was also increased by 3.5-fold under conditions of indole treatment (Fig. 1C). We call this behavior "rapid random twitching" (RRT).

Our previous studies demonstrated that indole signals can be recognized by the two-component system consisting of QseC, a sensor protein with histidine kinase activity, and QseB, a response regulator that modulates expression of downstream target genes (quorum-sensing *E. coli* regulators C and B). To determine whether the change in twitching activity described above was driven by the perception of the indole signal, we analyzed the twitching activity of *qseC* and *qseB* deletion (Δ*qseC*/*B*) mutants. The results showed that indole could no longer induce RRT in Δ*qseC* or Δ*qseB* mutants. In addition, the motility of both Δ*qseC* and Δ*qseB* mutants was significantly decreased in the presence or absence of indole (Fig. 1D). To exclude the possibility that this result was caused by a change in the mutants' growth speed, we monitored bacterial growth and found that the mutants grew similarly to the wild-type strain regardless of the presence or absence of indole (Fig. 1E). These results indicate that QseC/QseB-mediated indole signaling is involved in the control of twitching motility in *Lysobacter*.

Indole regulates twitching motility through the gene cluster *pilE1-pilY$_1$1-pilX1-pilW1-pilV1-fimT1*. To explore the mechanism of indole-driven RRT activity of *Lysobacter*, we analyzed the type IV pilin (Tfp) biosynthesis system of *L. enzymogenes* YC36. Tfp formation relies on 10 gene clusters (or genes) that are distributed in different genomic locations, namely, gene clusters *pilE1-pilY$_1$1-pilX1-pilW1-pilV1-fimT1*, *pilE2-pilY$_1$2-pilX2-pilW2-pilV2-fimT2-fimU*, and *pilG-pilH1-pilI-pilJ*, gene clusters *pilM-pilN-pilO-pilP-pilQ*, *pilT1-pilT2*, and *pilS1-pilR-pilA-pilB-pilC*, and solo genes *pilZ*, *pilH2*, *pilF*, and *pilS2* (Fig. 2A). Among them, indole significantly upregulated gene cluster *pilE1-pilY$_1$1-pilX1-pilW1-pilV1-fimT1* by 5-fold to 7-fold compared with untreated *L. enzymogenes* YC36 as shown by real-time quantitative PCR (qPCR), though the other 9 gene clusters were not affected by indole (Fig. 2B). Consistently, deletion of the *qseB* gene, which prevents the perception of indole signals, resulted in very weak expression of *pilE1-pilY$_1$1-pilX1-pilW1-pilV1-fimT1*. This result led us to speculate that indole regulates assembly of minor pilins through *qseC*/*B* and thus affects twitching motility behavior. To test this hypothesis, we first analyzed whether *pilE1-pilY$_1$1-pilX1-pilW1-pilV1-fimT1* is necessary or redundant for twitching motility. We knocked out the *pilW1* gene because this gene is key for this gene cluster and essential for fiber stability and function (40). According to the results, mutation of *pilW1* did not affect the growth of *L. enzymogenes* (see Fig. S1 in the supplemental material), while the mutant displayed significantly reduced twitching motility (Fig. 2C and D). Regardless of the presence or absence of indole, the twitching activity of the Δ*pilW1* mutant was only 20% of that of the wild-type strain (Fig. 2D). In addition, no RRT was observed, even in the presence of indole (Fig. 2E). Moreover, we adopted a double crossover strategy to replace the native promoter of the *pilE1-pilY$_1$1-pilX1-pilW1-pilV1-fimT1* gene cluster by the promoter of *groESL* and obtained the mutant *Le*YC36-P$_{groESL}$ (Fig. 2F). The *groESL* promoter was selected because previous studies have shown that *groESL* is expressed consistently under conventional culture conditions. Our results indicated that the *pilE1-pilY$_1$1-pilX1-pilW1-pilV1-fimT1* gene cluster was no longer positively regulated by exogenous indole and that the presence of indole did not cause the mutant to exhibit the RRT phenotype (Fig. 2G and H).

Intraspecies *Le*DSF and interspecies indole signals regulates the twitching motility of *Lysobacter*. In general, twitching motility is observed with high bacterial density, though relatively few studies of this type of social activity have been performed with low bacterial density or even with individual bacteria. In this study, we observed whether twitching motility appears at low concentrations of *L. enzymogenes* cells. The results showed that under conditions of extremely low density, individual bacterial cells grew and divided in a stable manner *in situ* without any movement Moreover, the twitching ability was not enhanced after indole was added (Fig. 3; see also Movies S3

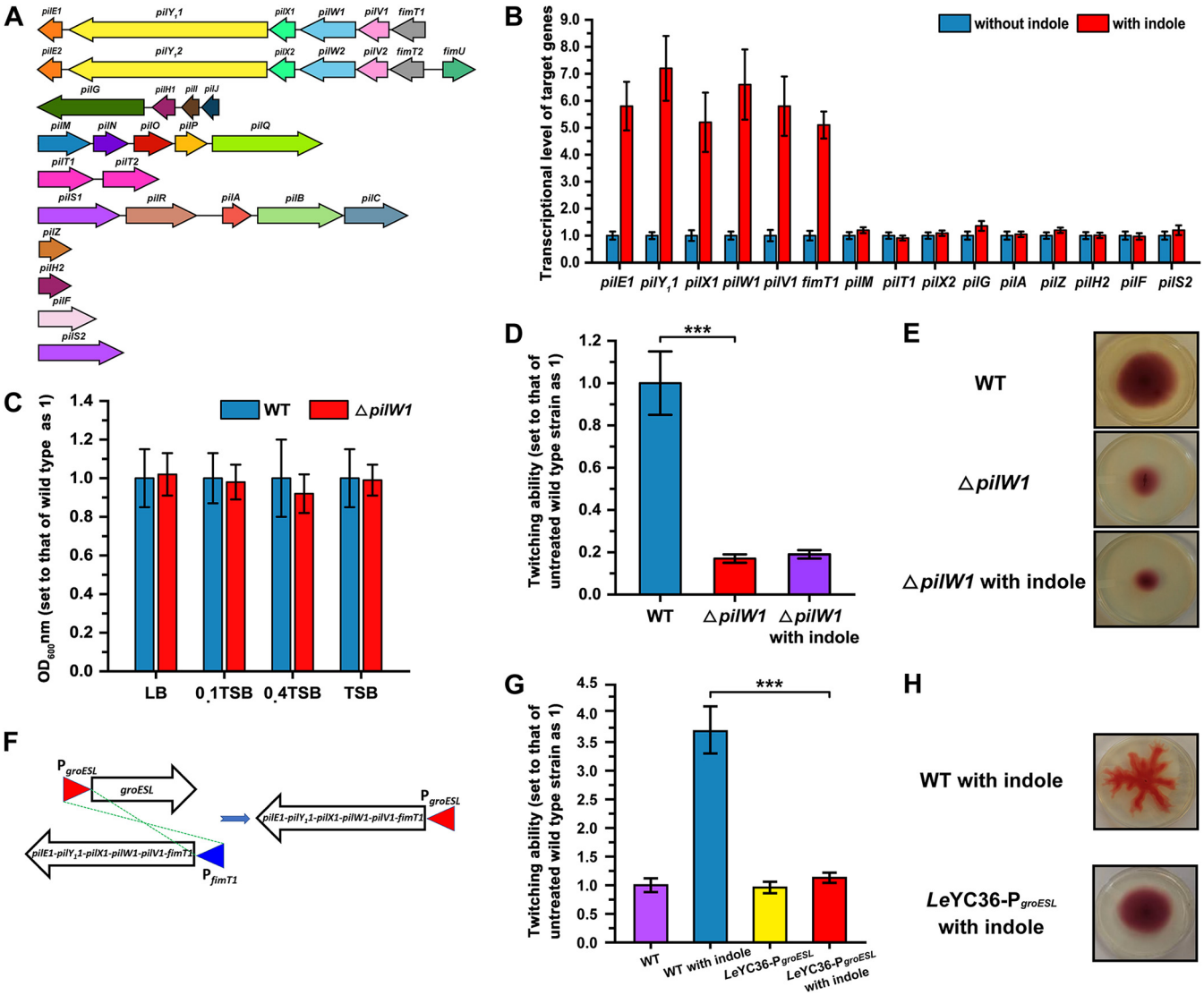

FIG 2 Indole regulates the twitching motility of *L. enzymogenes* through the gene cluster *pilE1-pilY*$_1$*1-pilX1-pilW1-pilV1-fimT1*. (A) Analysis of type IV pilin (Tfp) biosynthesis and assembly gene clusters of *L. enzymogenes* YC36. (B) The transcriptional levels of Tfp biosynthesis genes in the presence or absence of 0.5 mM indole. The value corresponding to the relative transcription level of untreated *L. enzymogenes* YC36 was set as 1. (C) OD_{600} analysis of the wild-type strain and Δ*pilW1* mutant in LB, 0.1 Trypticase soy broth (TSB), 0.4 TSB, and TSB media for 12 h of cultivation. The OD_{600} of wild-type *L. enzymogenes* YC36 was set as 1. (D) Analysis of twitching ability of the Δ*pilW1* mutant in the presence or absence of 0.5 mM indole. The twitching ability of the untreated wild-type strain was set as 1. (E) Comparison of the twitching motility patterns of the wild-type strain and Δ*pilW1* mutant in the presence or absence of 0.5 mM indole in motility test medium. To show the results clearly, we cultivated the strains for 72 h. The results are representative of biological duplicates. (F) Construction of mutant *Le*YC36-P_{groESL}. We replaced the promoter of the *pilE1-pilY*$_1$*1-pilX1-pilW1-pilV1-fimT1* gene cluster with the promoter of *groESL*. Sequencing verification of the mutant is provided in the supplemental material. (G) Analysis of twitching ability of the *Le*YC36-P_{groESL} mutant in the presence or absence of 0.5 mM indole. The twitching ability of the untreated wild-type strain was set as 1. (H) Comparison of the twitching motility patterns of the wild-type strain and Δ*pilW1* mutant in the presence of 0.5 mM indole in motility test medium. To show the results clearly, we cultivated strains for 72 h. The results are representative of biological duplicates. The error bars represent the standard deviations of results from at least three replicates. A *t* test of unpaired unequal variances was performed for testing differences between groups. For statistical analysis, ***, **, and * indicate $P < 0.001$, $P < 0.01$, and $P < 0.05$, respectively.

and S4). These results suggested that there are some other substances regulate the twitching ability of *L. enzymogenes* at low density.

Our findings prompted us to speculate that some quorum-sensing molecules are involved in population-dependent twitching motility. Therefore, we focused our attention on *Le*DSF, the only intraspecies quorum-sensing molecule identified in the *Lysobacter* genus so far. Our previous studies have shown that the *rpf* gene cluster, comprising *rpfB*, *rpfF*, *rpfG*, and *rpfC*, directs the biosynthesis and sensing of *Le*DSF and that loss of *rpfF* causes *Lysobacter* to lose the ability to produce *Le*DSF (31). Our results demonstrated that the twitching motility of the Δ*rpfF* mutant disappeared completely.

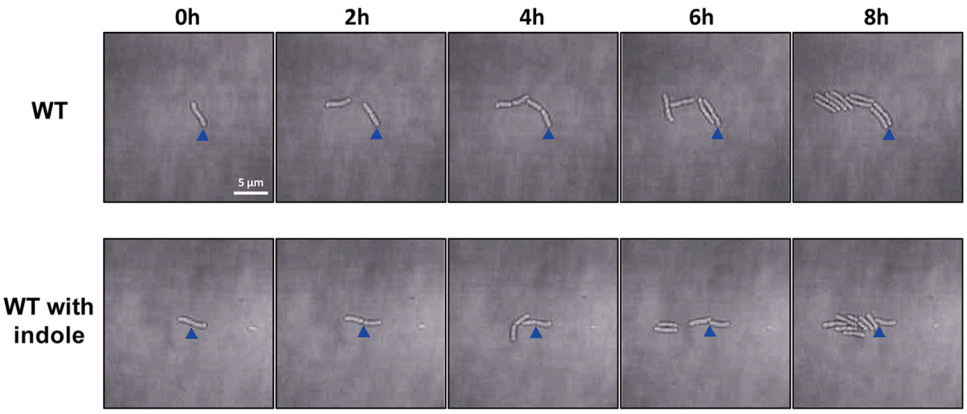

FIG 3 Dynamic imaging experiments assessing the twitching motility of individual bacterial cells in the absence or presence of 0.5 mM indole. The results showed that the isolated cells did not exhibit twitching motility regardless of the presence of indole. Each solid triangle indicates a fixed position reference. The magnification setting of the microscope was ×640.

Indeed, twitching motility and RRT behavior could not be restored, even in the presence of indole (Fig. 4A and B). To exclude the possibility that the observed behavior of the Δ*rpfF* mutant was caused by decreased growth capacity, we examined the growth of the mutant strain and found that *rpfF* knockout did not affect growth under conditions of exposure to various culture media (Fig. 4C; see also Fig. S1). When 1 μM exogenous *Le*DSF was added, the twitching motility of the Δ*rpfF* strain was restored. Moreover, the exogenous *Le*DSF-indole combination stimulated RRT formation in the Δ*rpfF* strain (Fig. 4A and B). Transcriptional analyses showed that deletion of *rpfF* reduced the expression level of the *pilM-pilN-pilO-pilP-pilQ* and *pilS1-pilR-pilA-pilB-pilC* gene clusters (Fig. 4D) and that expression of the two gene clusters was restored when the Δ*rpfF* mutant was complemented with ectopically expressed *rpfF* (Fig. 4E). The *pilE1-pilY*$_1$*1-pilX1-pilW1-pilV1-fimT1* gene cluster, which is related to assembly of minor pilins, was not affected by loss of *rpfF* (Fig. 4D and E). These results indicate that the synthesis and assembly of the Tfp system is regulated not by only one signaling molecule but by at least the interspecies indole and intraspecies *Le*DSF signaling molecules. Quorum-sensing molecules are produced only when the bacterial population reaches a certain density; low-density bacteria cannot *produce Le*DSF and therefore cannot generate twitching motility. Thus, we sought to determine whether exogenous *Le*DSF is able to endow low-density *Lysobacter* with twitching motility. Subsequently, 1 μM exogenous *Le*DSF was added to the culture medium, and obvious twitching activity was observed in individual cells, confirming our speculation. Moreover, under conditions in which the amount of *Le*DSF in the environment was insufficient to stimulate the quorum-sensing activities of the bacteria (0.1 μM), the single cells exhibited no twitching motility (37). Conversely, when *Le*DSF was present at greater than 1 μM, single *Lysobacter* cells displayed obvious, regular twitching motility, though an increase in the concentration of *Le*DSF did not enhance the speed of bacterial movement (Fig. 4F and G; see also Movie S5). This result suggests that when the concentration of the signal molecule *Le*DSF reaches a certain threshold, it can stimulate the Tfp system.

DISCUSSION

The molecular mechanisms and physiological functions of the interspecies signal indole in bacteria have been studied by several research groups. For example, early studies have illustrated that SidA, a DNA-binding transcriptional dual regulator, mediates indole signaling (41–43). In antibiotic-resistant bacteria, indole activates expression of several stress response genes and xenobiotic exporter genes, including *mdtA*, *mdtE yceL*, *cusB*, *acrD*, *acrE*, and *emrK* (28, 44). There is ample evidence showing that indole reduces persistence in *E. coli* (45–47). Furthermore, indole can induce the population-

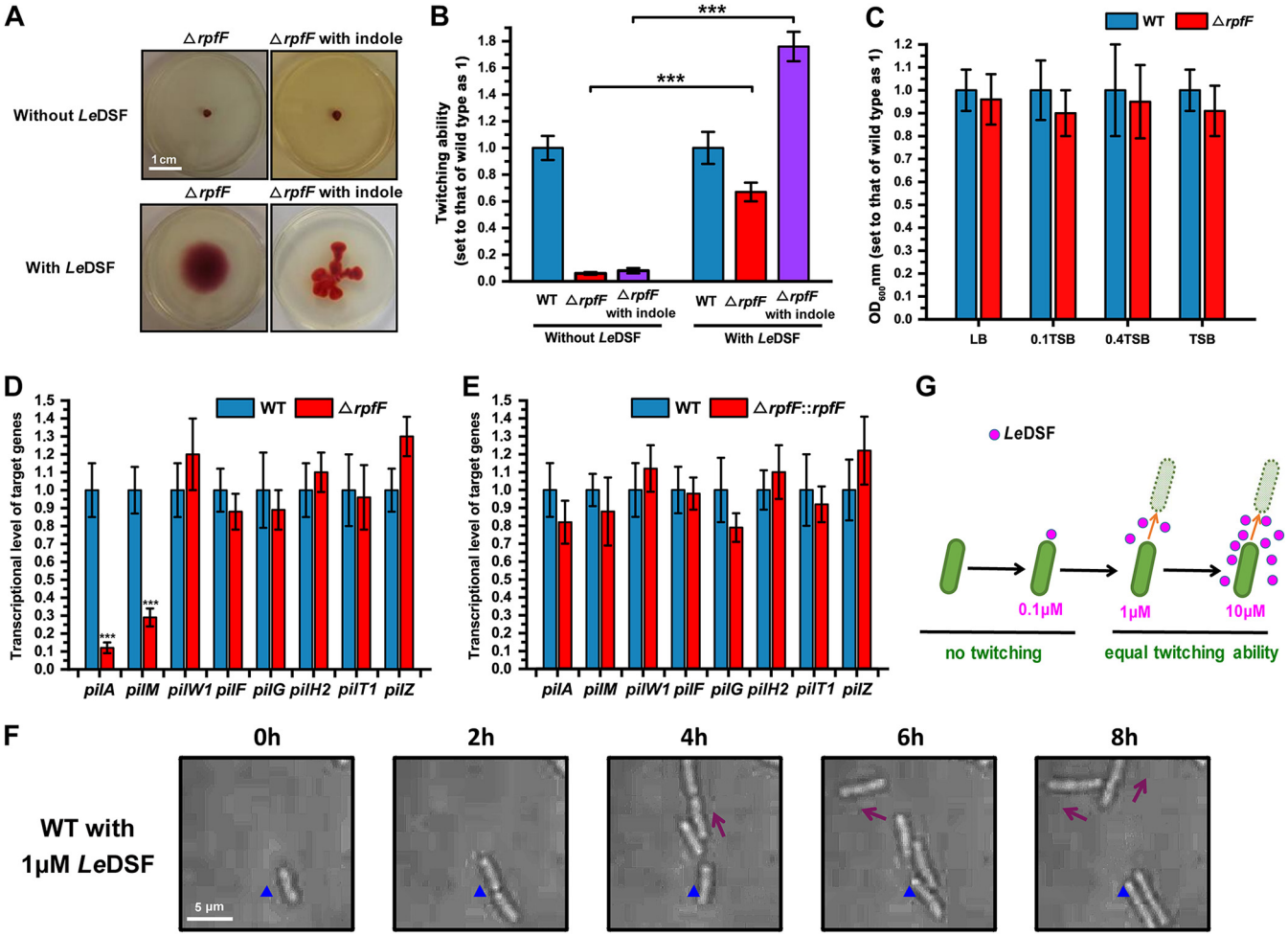

FIG 4 Intraspecies *Le*DSF regulates the twitching motility of *L. enzymogenes* by indirectly regulating gene clusters *pilM-pilN-pilO-pilP-pilQ* and *pilS1-pilR-pilA-pilB-pilC*. (A) Comparison of the twitching motility patterns of the Δ*rpfF* mutant in the presence or absence of 0.5 mM indole or 1 μM *Le*DSF in motility test medium. For this experiment, we cultivated strains for 48 h. The results are representative of biological duplicates. (B) Analysis of twitching ability of the Δ*rpfF* mutant in the presence or absence of 0.5 mM indole or 1 μM *Le*DSF. (C) OD_{600} analysis of the wild-type strain and Δ*rpfF* mutant in LB, 0.1 TSB, 0.4 TSB, and undiluted TSB media for 12 h of cultivation. The OD_{600} of the wild-type strain was set as 1. (D and E) The transcriptional levels of Tfp biosynthesis genes in Δ*rpfF* (D) and Δ*rpfF*::*rpfF* (E) mutants. The relative transcription level of untreated *L. enzymogenes* YC36 was set as 1. (F) Twitching motility of low-density, individual *L. enzymogenes* cells induced by exogenous 1 μM *Le*DSF. The results showed that individual cells exhibit twitching motility in the presence of 1 μM *Le*DSF. The magnification setting of the microscope was ×640. The solid triangle indicates a fixed position reference. The results are representative of biological duplicates. (G) A schematic diagram of the *Le*DSF-induced twitching motility of *L. enzymogenes* YC36. The error bars represent the standard deviations of results from at least three replicates. A *t* test of unpaired unequal variances was performed for testing differences between groups. For statistical analysis, ***, **, and * indicate $P < 0.001$, $P < 0.01$, and $P < 0.05$, respectively.

dependent antibiotic resistance of bacteria. A more recent study reported that indole signaling is sensed by *Lysobacter* through the *qseB/C* two-component system, thus reversing intrinsic multiantibiotic resistance (37). However, it remains largely unknown whether indole signaling affects other bacterial social behaviors and, if so, what mechanisms are involved.

Twitching motility is one of the major social activities of a wide range of bacteria, including *M. xanthus*, which exhibits complex social behaviors, and the opportunistic pathogen *P. aeruginosa*. The pilus is mainly composed of a combination of major pilins and low-abundance minor pilins. We show here that the interspecies signaling molecule indole is sensed by cells and then regulates gene cluster *pilE1-pilY*$_1$*1-pilX1-pilW1-pilV1-fimT1* of *L. enzymogenes* YC36 through the two-component system QseC/QseB and that intraspecies *Le*DSF regulates expression of gene clusters *pilM-pilN-pilO-pilP-pilQ* and *pilS1-pilR-pilA-pilB-pilC*. Why does *Lysobacter* use both intraspecies and interspecies signals to regulate twitching motility? We hypothesize that this strategy is

beneficial to environmental adaptation in bacteria. When there are no intraspecies signals or interspecies signals in the environment, *Lysobacter* cells are aware that there is no competitive pressure and that they do not need to improve their survival abilities through social behaviors, such as twitching motility. However, when the intraspecies signal *Le*DSF appears in the environment, *Lysobacter* can perceive that there is more competition, and it becomes necessary for cells to communicate with each other through regular twitching motility. When the environment has both the intraspecies signal *Le*DSF and the interspecies signal indole, a given *Lysobacter* cell can recognize the surrounding *Lysobacter cells* and other bacterial species, causing *Lysobacter* to initiate a series of physiological behaviors, including rapid random twitching motility, to adapt to the changing external environment and to invoke competitive behaviors (15, 16).

Minor pilins are important players in the biogenesis and function of Tfp in several bacterial species, including *E. coli*, *M. xanthus*, and *P. aeruginosa* (48–55) In *P. aeruginosa*, it has been shown that minor pilins are positively regulated by the two-component system FimS-AlgR (40). However, whole-genome sequencing of *L. enzymogenes* YC36 showed that the strain did not possess the FimS-AlgR two-component system. Thus, how gene cluster *pilE1-pilY*$_1$*1-pilX1-pilW1-pilV1-fimT1* is regulated in *Lysobacter* is an interesting issue. In this study, we found that the two-component system QseB/C regulates gene cluster *pilE1-pilY*$_1$*1-pilX1-pilW1-pilV1-fimT1*. Nonetheless, it remains unclear whether QseB directly regulates gene cluster *pilE1-pilY*$_1$*1-pilX1-pilW1-pilV1-fimT1* or regulates those genes indirectly by regulating other regulatory genes, such as the global regulatory factor CLP gene. This issue needs to be addressed in a follow-up study.

Compared with regulation of indole-induced gene cluster *pilE1-pilY*$_1$*1-pilX1-pilW1-pilV1-fimT1*, regulation of gene clusters *pilM-pilN-pilO-pilP-pilQ* and *pilS1-pilR-pilA-pilB-pilC* has been clarified through this study and previously published research. For example, the Qian group showed that the Clp global regulator has a dual role in controlling twitching motility in *L. enzymogenes* OH11 (22). Clp directly controls transcription of *pilA* and *pilM-pilN-pilO-pilP-pilQ* by binding to their promoter regions. This finding suggests that *Le*DSF does not directly regulate major pilin biosynthesis and assembly genes but modulates them indirectly through RpfC and RpfG, leading to a reduction in cyclic di-GMP levels. The absence of *rpfF* leads to the absence of *Le*DSF and to elevations in the cellular level of cyclic di-GMP (56); thus, inactivated Clp can no longer activate the *pilM-pilN-pilO-pilP-pilQ* and *pilS1-pilR-pilA-pilB-pilC* gene clusters (Fig. 5). Overall, the abundance of Clp directly controls gene clusters *pilM-pilN-pilO-pilP-pilQ* and *pilS1-pilR-pilA-pilB-pilC*.

The twitching motility of *L. enzymogenes* is closely associated with the attachment and colonization of fungal mycelia (57). Thus, Tfp formation is recognized as a potential antifungal strategy employed by *Lysobacter*. As *Lysobacter* species also produce multiple bioactive natural products and the regulatory mechanism remains largely unexplored, it will be interesting to explore whether indole/*Le*DSF-induced twitching motility also plays a role in regulating antibiotic production in *Lysobacter*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and general methods. *Lysobacter* strains were grown on lysogeny broth medium (LB) or 40%-strength tryptic soy broth (TSB; Sigma) (1/10) at 30°C (58). *E. coli* DH5α was grown in LB medium and used as the host for DNA manipulation. When required, the following antibiotics were used: gentamicin, 40 mg/ml; ampicillin, 100 mg/ml; kanamycin, 50 mg/ml. The concentration of indole in all experiments was 0.5 mM. Genomic DNA of *L. enzymogenes* was prepared as previously described (59). Plasmid construction and related DNA fragment extractions were performed following the instructions provided with kits purchased from Omega (plasmid minikit I and gel extraction kit; Omega, USA). Restriction enzymes and other molecular biology materials were purchased from TaKaRa (TaKaRa Bio Group, Japan). PCR primers were synthesized by Tsingke Biological Technology Company (Qingdao, China). The bacterial strains and plasmids used in this study are described in Table 1. The primers used are described in Table 2.

Twitching motility test for *L. enzymogenes*. The motility test medium contained 1% peptone, 0.3% beef extract, 0.5% NaCl, and 0.4% agar. To facilitate visual observation of the motility of *L. enzymogenes*, 2,3,5-triphenyltetrazolium chloride (1% TTC; 5 ml) was added to the medium (1 liter) as a color indicator

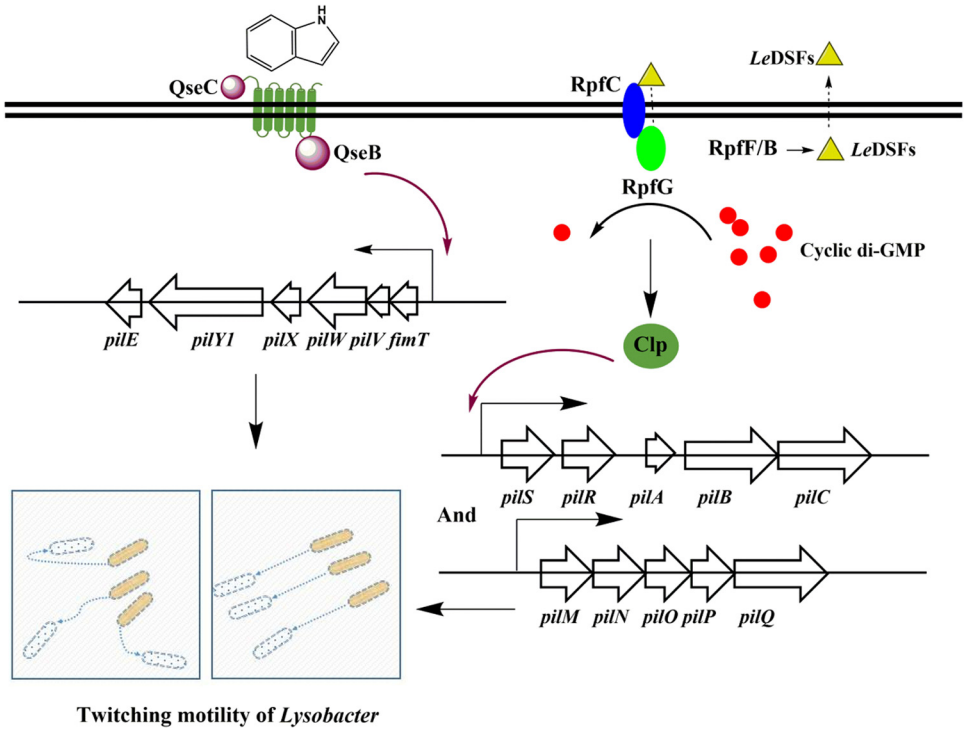

FIG 5 Schematic diagram of the synergistic regulation of the twitching motility of *L. enzymogenes* by the intraspecies signal *Le*DSF and the interspecies signal indole. Reduction in cyclic di-GMP levels through activation of RpfG allows Clp to bind to the promoters of the *pilM-pilN-pilO-pilP-pilQ* and *pilS1-pilR-pilA-pilB-pilC* gene clusters. Whether QseB directly regulates expression of *pilE1-pilY*$_1$*1-pilX1-pilW1-pilV1-fimT1* is unknown.

(60). As the bacterial cells grew, the colorless TTC was converted into reddish TPF by the reductase activities of the bacterial cells. Each of the strains was inoculated at the center of the plate using a sterile toothpick to penetrate the 3-mm-thick solid motility test medium, and the plates were placed in a 28°C incubator for 2 to 3 days. An indirect measurement assay was performed to calculate the twitching speed of the bacteria. Bacterial movement can be observed by continuous photography under a microscope for 30 h. The bacteria were randomly selected for twitching speed calculation according to their movement distance at a specific time point under the microscope. In this experiment, we set the value representing the twitching speed of untreated bacteria to 1.

RNA extraction and qPCR. Wild-type and mutant strains of *L. enzymogenes* YC36 were grown in 100 ml 40% TSB medium *for 36 h*. A 3-ml aliquot of cells was transferred to sterile tubes and centrifuged for 5 min at 15,000 × *g*. TRIzol solution was used to isolate total RNA from the cells following the manufacturer's instructions. A PrimeScript reverse transcription reagent kit was used with a genomic

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source (reference)
Strains		
L. enzymogenes YC36	Wild-type strain	This study
Escherichia coli DH5α	Competent cells that can be used for DNA manipulation	This study
Escherichia coli S17-1	RP4-2Tc::Mu-Kn::Tn*7 pro hsdR recA*; host for required plasmids; conjugal donor	This study
Escherichia coli Δ*pilW*	*pilW* gene deletion mutant strain	This study
Escherichia coli Δ*pilW*::*pilW*	*pilW* complementary strain of the Δ*pilW* mutant	This study
Escherichia coli Δ*rpfF*	*rpfF* gene deletion strain	Laboratory of L. Du (31)
Escherichia coli Δ*rpfF*::*rpfF*	*rpfF* complementary strain of the Δ*rpfF* mutant	This study
Escherichia coli Δ*qseB*	*qseB* gene deletion strain	Laboratory of L. Du (36)
Escherichia coli Δ*qseC*	*qseC* gene deletion strain	Laboratory of L. Du (36)
Plasmids		
pEX18	Gm^r; *oriT*$^+$ *sacB*$^+$, gene replacement vector with MCS from pUC18[a]	This study
pEX18-T	Used for gene deletion; pEX18 carrying the *orf* gene from *L. enzymogenes*	This study
pHmgA-P	Contains promoter of HSAF and selection marker (used for target gene complementation)	Laboratory of L. Du (71)
pHmgA-P-G	The complementary gene was linked to pHmgA-P and formed pHmgA-P-G; this plasmid was used for expression of the target gene	This study

[a]Gm^r, gentamycin resistance.

TABLE 2 Primers used in this study

Purpose	Primer	Sequence
qPCR assay	*pilE*-up	5′-AGGCCAAGGCCGATCTGGC-3′
	pilE-down	5′-GATGGTCAGGTTCTTGCAC-3′
	pilY1-up	5′-AACGGCAGCGTGCTGACCT-3′
	pilY1-down	5′-AGCTCTTCCTTCAGGCGCG-3′
	pilX-up	5′-ACGCATGTCGACCAATTAC-3′
	pilX-down	5′-TGCCGACCTTGATGCTGGA-3′
	pilW-up	5′-CTGACCAATCCGGCCGGCT-3′
	pilW-down	5′-CAGCACGACGACGTCGCTG-3′
	pilV-up	5′-AACTACCGCACCCGCGCCA-3′
	pilV-down	5′-GCTGCGGCGGACGATCGC-3′
	fimT-up	5′-TGGCCCTGCCCAGTTTCAC-3′
	fimT-down	5′-CACGCTCTCGGTGCGGC-3′
	pilM-up	5′-CGGCATGCCGGCCGATCT-3′
	pilM-down	5′-AGATCGGCCGGCATGCCG-3′
	pilT-up	5′-GGTCGAAGACCCGATCGA-3′
	pilT-down	5′-GATGCGGTCCACGGTCT-3′
	$pilX_2$-up	5′-GAACTACGTGCCGTATCCGATC-3′
	$pilX_2$-down	5′-GTCATGCGCCGCTACGGAGCG-3′
	pilG-up	5′-ATCGACGGATTCGAGGCGT-3′
	pilG-down	5′-GCGGATCGCGTCGAGGAGC-3′
	pilA-up	5′-TCGCCGAGTCGGCCAACGA-3′
	pilA-down	5′-GCGGCAGGACGACGGGAT-3′
	pilZ-up	5′-TACATGCCGTTCCTGAAGTA-3′
	pilZ-down	5′-TCAACGTGCCCGCCAGCAG-3′
	$pilH_2$-up	5′-AGAAGACGGCGCCGCCGGC-3′
	$pilH_2$-down	5′-AGGGCAGCAACTGGCCGAT-3′
	pilF-up	5′-ACGATTTCAAGAACGACCA-3′
	pilF-down	5′-GTTGAGCGCCGCGCCGCGT-3′
	$pilS_2$-up	5′-CGCAGCCTGGCCGGCCAC-3′
	$pilS_2$-down	5′-AGCCGGCGATGAAGCGCT-3′
Construction of vectors for gene disruption	*pilW*-up	5′-TGGGGTACCACCGTTCCGACCG-3′
	pilW-down	5′-TAGGAATTCAACTGGTCGCGGGCA-3′
Construction of vectors for gene in-frame deletion	*pilW*-1-up	5′-CGGGATCCACGCCGATGCCGAGCAACA-3′
	pilW-1-down	5′-GCTCTAGACGCCACGGATCGCTCCGT-3′
	pilW-2-up	5′-GCTCTAGAATCTTGCCGTTGTAGTAG-3′
	pilW-2-down	5′-CCCAAGCTTTGCGCAAGTTCGAACTGT-3′
Mutant verification	*pilW*-V-up	5′-CCTATGGTTGCGATGGCTCA-3′
	pilW-V-down	5′-GCAAGGCGATTAAGTTGGGTA-3′
Gene complementation	*rpfF*-C-up	5′-CGGGATCCATGAGCACCATCGAAA-3′
	rpfF-C-down	5′-CCGCTCGAGTTACGCGGCCACGGC-3′
Construction of mutant *Le*YC36-P_{groESL}[a]	P_{fimT1}-1-up	5′-CGGAATTCGTCGCACAGCAGCCAGGG-3′
	P_{fimT1}-1-down	5′-GGGGTACCGCTTATGCGAATCAAGAC-3′
	P_{groESL}-1-up	5′-GGGGTACCGAAGGCCTTCCTCAGCCG-3′
	P_{groESL}-1-down	5′-CGGGATCCGGCGACCTCTGTAAGTAAT-3′
	P_{fimT1}-2-up	5′-CGGGATCCATGAGCAGGCGGCGAACTG-3′
	P_{fimT1}-2-down	5′-CCCAAGCTTGAAATCGCGCTTGCCGCCT-3′

[a]We replaced the promoter of the *pilE1-pilY*$_1$*1-pilX1-pilW1-pilV1-fimT1* gene cluster with the promoter of *groESL*.

DNA (gDNA) eraser kit (TaKaRa Biotechnology) to remove DNA and to reverse transcribe the mRNA. qPCR was then carried out in a total reaction volume of 20 μl containing 200 nM primers, 0.5 μl of diluted cDNA template (0.5 μl of RNA sample was used as a negative template to ensure that DNA contamination was removed), 10 μl of EvaGreen 2× qPCR Master Mix and 8.5 μl of RNase-free double-distilled water (ddH_2O). 16S rRNA was used as the reference gene. qPCR was performed using a StepOne qPCR system (AB Applied Biosystems). The qPCR program was designed as described previously (61). All experiments were repeated three times.

Bioinformatics analyses and gene information. Genomic DNA of our *Lysobacter enzymogenes* YC36 strain (GenBank accession number CP040656) was extracted according to a procedure previously described by Moore et al. (62), and the DNA G+C content was determined by whole-genome sequencing. The genome sequencing of strain *L. enzymogenes* YC36 was performed on an Illumina HiSeq platform (Illumina, USA). SOAPdenovo assembler software was applied to assemble these reads (63) (http://sourceforge.net/projects/soapdenovo2/files/SOAPdenovo2/). Putative coding sequences (CDSs)

were identified by Glimmer 3.0 (64). RNAmmer (65) and tRNAscan (66) were used to predict rRNAs and tRNAs, respectively, and small RNA (sRNA) was identified using the Rfam database (67). The annotation and bioinformatics analyses of this genome were carried out by the use of the RAST server (68) (http://rast.nmpdr.org/) and EMBOSS (The European Molecular Biology Open Software Suite) (http://emboss.open-bio.org/).The annotated sequences of the twitching-motility-related genes were checked by BLAST with the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The primers for qPCR and the gene manipulation assays were designed using Primer Premier 5 (69). ENDscript 2 software was used to compare the target proteins (70). The primers used for qPCR are listed in Table 2. Gene information and GenBank accession numbers of the Tfp assembly system of *L. enzymogenes* YC36 are listed in Table S1 in the supplemental material.

Twitching-motility detection under a microscope. All images were captured under an inverted microscope. A FCS2 flow cell system (Bioptechs) was used to record time-lapse images. The cells were cultured, collected, washed three times with 40% TSB medium, and imaged on a gel pad that contained 2% low-melting-temperature agarose. *L. enzymogenes* cells were observed at 30°C. An indirect measurement assay was performed to calculate the twitching speed of the bacteria. Bacterial movement can be observed by continuous photography under a microscope for 30 h. The bacteria were randomly selected for twitching speed calculation according to their movement distance (measured in micrometers) at a specific time point under the microscope. The magnification setting of the microscope was ×640.

Generation of gene deletion, promoter replacement, and gene complemented mutants. To construct vectors for gene deletion in *L. enzymogenes* YC36, upstream and downstream fragments were amplified using the primer pairs listed in Table 2. Genomic DNA was used as the PCR template. The upstream and downstream fragments of each gene were cloned into pEX18 to generate pEX18-G. The resulting plasmids were transferred into *L. enzymogenes* via conjugation according to a method described previously (59). Confirmed colonies were subjected to a second round of crossover to produce the gene deletion mutants. Target colonies were selected by PCR and verified by sequencing. Similarly, the promoter replacement experiment was performed using a double-crossover strategy and the same vector as described above. The only difference was the addition of P_{groELS} promoter sequences between the upstream and downstream homologous arms. (Please see Table 2 for primer information.) pHmgA-P was used for a gene complement assay, with the complete gene sequence amplified and linked to the vector to generate pHmgA-P-G. pHmgA-P-G plasmids were transferred into *L. enzymogenes* by conjugation according to a previously described method (71).

Accession number(s). Newly determined sequence data were deposited in GenBank under accession numbers MK967260 to MK967276.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01742-19.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.
SUPPLEMENTAL FILE 2, MP4 file, 0.6 MB.
SUPPLEMENTAL FILE 3, MP4 file, 0.6 MB.
SUPPLEMENTAL FILE 4, MP4 file, 0.3 MB.
SUPPLEMENTAL FILE 5, MP4 file, 0.2 MB.
SUPPLEMENTAL FILE 6, MP4 file, 0.3 MB.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (no. 31870023, 31571970, and 41506160), the Fundamental Research Funds for the Central Universities (no. 201941009), the Young Elite Scientists Sponsorship Program by CAST (no. YESS20160009), and the Marine S&T Fund of Shandong Province for Pilot National Laboratory for Marine Science and Technology (Qingdao) (no. 2018SDKJ0406-4).

Y.W. and Y.H. conceived the project. Y.W., L.D., and Y.H. designed the experiments. T.F., Y.H., B.L., Z.L., Y.Y., X.L., and Q.S. carried out experiments. Y.W., T.F., and Y.H. analyzed the data. Y.W. wrote the manuscript draft. L.D. and T.F. revised the manuscript. All of us read and approved the submission for publication.

We declare that we have no conflicts of interest.

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