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ABSTRACT

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Lysobacter enzymogenes strain C3 is a gliding bacterium which produces the antifungal secondary metabolite heat-stable antifungal factor (HSAF) and type IV pilus (T4P) as important mechanisms in biological control activity against fungal pathogens. To date, the regulators that control HSAF biosynthesis and T4P-dependent twitching motility in *L. enzymogenes* are poorly explored. In the present study, we addressed the role of *pilG* in the regulation of these two traits in *L. enzymogenes*. PilG of *L. enzymogenes* was found to be a response regulator, commonly known as a component of a two-component transduction system. Mutation of *pilG* in strain C3 abolished its ability to display spreading colony phenotype and cell movement at the colony margin, which is indicative of twitching motility; hence, PilG positively regulates twitching motility in *L. enzymogenes*. Mutation of *pilG* also enhanced HSAF production and the transcription of its key biosynthetic gene *hsaf pks/nrps*, suggesting that PilG plays a negative regulatory role in HSAF biosynthesis. This finding represents the first demonstration of the regulator PilG having a role in secondary metabolite biosynthesis in bacteria. Collectively, our results suggest that key ecological functions (HSAF production and twitching motility) in *L. enzymogenes* strain C3 are regulated in opposite directions by the same regulatory protein, PilG.

Lysobacter enzymogenes is a soil-inhabiting, gram-negative bacterial species with strains that have shown biological control activity against fungal and oomycetous pathogens (Folman et al. 2004; Giesler and Yuen 1998; Qian et al. 2009; Sullivan et al. 2003). The species is known to be versatile in production of antimicrobial lytic enzymes and secondary metabolites (Christensen and Cook 1978; Kobayashi and Yuen 2007; Xie et al. 2012). Evidence has been found for both types of antimicrobial compounds having roles in antagonism and biological control against fungi (Folman et al. 2004; Kobayashi et al. 2005; Li et al. 2008; Palumbo et al. 2005), with production of one secondary metabolite, a heat-stable antifungal factor (HSAF), being demonstrated definitively to be an important mechanism in the biocontrol of certain fungal diseases (Li et al. 2008). HSAF was characterized structurally be a complex containing dihydromaltophilin and structurally related macro-cyclictetramic acids (Yu et al. 2007). This antifungal compound has been the subject of a number of studies, including the identification of its unique biosynthetic pathway (Lou et al. 2011) and its effects on hyphal growth and development (Li et al. 2006, 2008).

Pathogenesis on hyphae also is regarded as a mechanism of biocontrol against fungi by *L. enzymogenes* (Mathioni et al. 2013; Patel et al. 2013). In this regard, the ability of *L. enzymogenes* to attach to and infect fungal hypha was reported to be dependent on the production of type IV pilus (T4P), which is a thin, hair-like appendage formed from pilin, or PilA, subunits (Patel et al. 2011). Gliding or surface motility, another notable phenotype in *L. enzymogenes* (Christensen and Cook 1978), also was shown to be dependent on T4P (Patel et al. 2010; Wang et al. 2014). Thus, the term "twitching motility" should be applied (Mattick 2002). Although the importance of twitching motility to biological control activity of *L. enzymogenes* has not been investigated, twitching motility is thought to be important in the colonization of host surfaces and production of biofilms (Mattick 2002), processes that are considered to be critical to the establishment of bacteria in general on plant surfaces (Burdman et al. 2011).

The molecular mechanisms that regulate these biocontrol-important traits are only beginning to be understood. In an early study, the production of excreted antifungal factors by *L. enzymogenes* strain C3, as well as twitching motility, was shown to be dependent on signaling by a cyclic adenosine monophosphate (cAMP)-receptor-like protein (Clp), with mutant strains affected in *clp* being deficient in both activities (Kobayashi et al. 2005). Using *L. enzymogenes* strain OH11, we found that a functional Clp is required for HSAF biosynthesis and twitching motility but Clp regulation of the two processes is independent of one another (Wang et al. 2014). In a separate study, we found that two distinct quorum-sensing systems upregulate HSAF production but, whereas the system mediated by the diffusible signal factor (DSF) blocked the transcription of motility-related genes, the other, mediated by the diffusible factor (DF), had no effect on that group of genes (Qian et al. 2013). Findings from these studies are only suggestive of the many complex systems that might be involved in regulating these biocontrol-related traits in *L. enzymogenes*.

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X. Zhou and G. Qian contributed equally to this study.

In surveying a draft genomic sequence of *L. enzymogenes* OH11, a strain isolated in China, we found that the genome possessed a homolog of *pilG*, first reported in *Pseudomonas aeruginosa* (Darzins 1993; Lou et al. 2011). We then defined the PilG homolog as PilG$_{Le}$ in *L. enzymogenes*. PilG of *P. aeruginosa* is a response regulator homologous to CheY in *Escherichia coli*, and has a phosphoryl receiver (REC) domain involved in receiving a signal from a sensor protein (Darzins 1993). Recently, PilG was shown to be a member of the large general type II secretory pathway (Gsp)F family of integral inner membrane proteins, which includes proteins involved in T4P biogenesis and type II secretion (Collins et al. 2007). PilG in *P. aeruginosa* is part of a chemosensory system that regulates the biosynthesis and the extension and retraction of T4P (Bertrand et al. 2010; Mattick et al. 1996; Whitchurch et al. 2004). In addition to regulation of T4P biogenesis and T4P-mediated cell motility, the other characterized roles of *pilG* in diverse bacteria such as *P. aeruginosa* and *Neisseria* spp. center on virulence via attachment to host cells and on competence for DNA uptake, both involving T4P (Bertrand et al. 2010; Tønjum et al. 1995; Whitchurch et al. 2004; Yoshihara et al. 2002). As noted above, the PilG regulator modulates twitching motility in *P. aeruginosa*; therefore, our goal was to investigate the role of PilG$_{Le}$ in T4P-dependent twitching motility in *L. enzymogenes* strain C3. Meanwhile, to our knowledge, the involvement of PilG in regulating production of antimicrobial secondary metabolites has not been demonstrated. In the present work, we also planned to explore whether or not PilG$_{Le}$ is involved in HSAF production by that strain for expanding the potential new regulatory roles in secondary-metabolite biosynthesis in bacteria. Here, we show that mutation of *pilG$_{Le}$* in strain C3 abolished twitching motility, suggesting that the role of PilG in controlling T4P-dependent cell motility is conserved between *L. enzymogenes* and other bacterial species (e.g., *P. aeruginosa*). Importantly, we demonstrate that inactivation of *pilG$_{Le}$* significantly enhanced the production of HSAF in comparison with the wild-type C3, which represents the first demonstration of the PilG proteins having a regulatory role in secondary metabolite biosynthesis in bacteria. Finally, we discuss the potential relationships between PilG, Clp, and DSF quorum sensing in the regulation of HSAF biosynthesis and twitching motility in *L. enzymogenes*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strain DH5α was used for plasmid constructions and was grown in Luria-Bertani (LB) broth at 37°C. Unless otherwise stated, *L. enzymogenes* wild-type strain C3 and its derivatives were grown in 10% tryptic soy broth (TSB) medium at 28°C. When required, antibiotics were added to the medium at the following final concentrations: kanamycin (Km) at 25 μg/ml, and gentamicin (Gm) at 25 and 150 μg/ml for *E. coli* and *L. enzymogenes*, respectively.

Generation of *pilG$_{Le}$* in-frame deletion mutant and its genomic integrated complemented strain in *L. enzymogenes*. *L. enzymogenes* wild-type strain C3 was used as a parent strain for generation of gene in-frame deletion mutants, as described previously (Qian et al. 2012). The scheme of construction of deletion mutant was shown in Supplementary Figure S1. The primers used here were listed in Supplementary Table S1. In brief, a pair of primers (1909F-1/1909F-2) was used to amplify a 656-bp upstream homolog arm of *pilG$_{Le}$*. The primer pair 1909R-1/1909R-2 was used to amplify a 597-bp downstream homolog arm of *pilG$_{Le}$*. These two homolog arms were cloned into the *Kpn*I/*Hin*dIII sites of the suicide vector pEX18GM (Table 1), resulting in a final construct. This final construct was transformed into the *Lysobacter* wild-strain cells by electrotransformation, as described previously (Qian et al. 2012). Then, *Lysobacter* transconjugants were selected on LB plates without sucrose and with Km (50 μg/ml) and Gm (150 μg/ml). The positive

colonies were plated on LB plates supplemented with 10% (wt/vol) sucrose and Km (50 μg/ml) to screen for resolution of the construct by a second cross-over event. The resultant mutants were validated by a polymerase chain reaction (PCR) method using the primers C1/C2, which was located in the outside of the manipulated region in the genome. All the DNA fragments were sequenced for their correction. Due to lack of a plasmid-based complementation system for strain C3 (Kobayashi et al. 2005), a genomic integration method based on homologous recombination was applied to construct the complemented strains of the *pilG$_{Le}$* mutants in the present study. The corresponding scheme was shown in Supplementary Figure S2. In brief, *pilG$_{Le}$*, together with its predicted promoter, was amplified from strain C3 by PCR with the designated primers and cloned into the suicide vector pEX18Gm to create the construct (Table 1). The final construct was transformed into the *pilG$_{Le}$* mutant for generating the genomic integrated complemented strain. The generated mutants and complemented strains were verified by PCR with a set of primers.

Observation of twitching motility. Twitching motility of the wild-type C3 of *L. enzymogenes* was first evaluated on three different media (water agar, 5% tryptic soy agar [TSA], and 100% TSA) under hard (1.8%) and soft (0.5%) agar conditions to determine the best sets of conditions for twitching motility. After screening, the 5% TSA with 1.8% agar subsequently was used in comparing twitching motility of mutant strains with wild-type strain C3. The method described previously (Wang et al. 2014) was used in microscopic observation of twitching motility. In brief, a thin layer of an agar medium (1 ml) was applied to a sterilized microscope slide and allowed to harden as a thin film. To create a thin inoculation line, the edge of a sterilized coverslip was dipped into a cell suspension of the bacterial strain and then gently pressed onto the surface of the agar. After 24 h of incubation, the margin of the bacterial culture on the microscope slide was observed under a compound microscope at greater than ×400 magnification without a coverslip. Twitching motility was evident in the form of individual bacterial cells or small clusters of cells growing separately from the main colony (Reichenbach 2006). Three replicate slides for each treatment were used, and the experiment was performed three times.

Extraction and quantitative analysis of HSAF. The wild-type C3 of *L. enzymogenes* and its derivatives were cultivated in 10% TSB for 2 days. Then, HSAF was extracted from these various *L. enzymogenes* cultures and detected by high-performance liquid

TABLE 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Characteristics[a]	Source
Lysobacter enzymogenes		
C3	Wild type, Km^R	Giesler and Yuen 1998
Δ*pilG*	*pilG* in-frame deletion mutant, Km^R	This study
Δ*pilG*cp	Genomic integrated complementary strain of the *pilG mutant*, Km^R, Gm^R	This study
OH11	Wild-type, Km^R	Qian et al. 2009
Δ*rpfF*$_{OH11}$	*rpfF*$_{OH11}$ in-frame deletion mutant, Km^R	Qian et al. 2013
Escherichia coli		
DH5α	*supE44lacU169 (ΔlacZΔM15) hsdR17 recA lendA1gyrA96 thi-1 relA11*	Lab collection
Plasmid		
pEX18GM	Suicide vector with a *sacB* gene, Gm^R	Hoang et al. 1998
pEX18GM-pilG	pEX18GM with two flanking fragments of *pilG*, Gm^R	This study
pEX18GM-pilGcp	pEX18GM cloned with a 570-bp fragment containing intact *pilG* and its predicted promoter, Gm^R	This study

[a] Km^R and Gm^R = kanamycin and gentamicin resistant, respectively.

chromatography (HPLC), as described previously (Qian et al. 2013, 2014; Yu et al. 2007). In the HPLC analysis, standard HSAF (Yu et al. 2007) were used as a control. Three replicates were used for each treatment, and the experiment was performed three times.

Detection of growth curve. Various *Lysobacter* strains, including the wild-type C3, the *pilG$_{Le}$* mutant, and the complemented strain of the *pilG$_{Le}$* mutant, were cultured in LB medium at 28°C overnight. The overnight culture (500 µl) for each strain was transferred into 50 ml of fresh 10% TSB to grow until the cell density, expressed by the optical density at 600 nm (OD$_{600}$), reached 1.0. Then, 1 ml of each culture was transferred again into the fresh 10% TSB (50 ml) to start the detection of the growth curve. All inoculation broths were grown at 28°C with shaking at 200 rpm, and the OD$_{600}$ value was determined every 2 or 4 h until bacterial growth reached the stationary stage. Each sample involved three technical replicates and the experiment was performed three times.

Quantitative reverse-transcription PCR. The wild-type C3 of *L. enzymogenes* and its derivatives were cultivated in 10% TSB until the cell density expressed by OD$_{600}$ reached 1.0, based on the growth curve of each strain. Total RNA from the cells of each culture was extracted using a bacterial RNA kit (number R6950-01; OMEGA) following the manufacturer's instructions. Then, the cDNA was synthesized using PrimerScript RT reagent Kit with gDAN Eraser (number RR047A; TaKaRa). The following quantitative reverse-transcription (qRT)-PCR assays were run on an ABI PRISM7500 machine using the kit SYBR Premix Ex Taq Plus with Tli RnaseH (number RR420A; TaKaRa), as described previously (Qian et al. 2013). Primer sequences used in this assay are listed in Supplementary Table S1. The 16s ribosomal RNA gene was chosen for a housekeeping gene or an internal control in this study, which was described previously in *Lysobacter* spp. (Qian et al. 2013, 2014).

Data analysis. All analyses were conducted using SPSS 14.0 (SPSS Inc.). The hypothesis test of percentages (*t* test, *P* = 0.05 or 0.01) was used to determine significant differences in the production of HSAF and gene expressions between the wild-type C3 and its derivatives.

RESULTS

Identification and sequence characterization of PilG$_{Le}$ in *L. enzymogenes*. To examine whether the genome of *L. enzymogenes* contains a potential gene that encodes a PilG protein, the well-studied PilG of *P. aeruginosa*, with a size of 117 amino acids (aa) (GI: 15595605), was selected as the template to perform a BlastP search in the available draft genome of *L. enzymogenes* strain OH11 (Lou et al. 2011). This led to the identification of a corresponding ortholog, also termed PilG$_{Le}$ in *L. enzymogenes*. PilG$_{Le}$ (117 aa) shared 92% identity at the amino-acid level (e value = 1e^{-73}) with the counterpart in *P. aeruginosa* (Fig. 1). Domain analysis showed that PilG$_{Le}$ contained a conserved REC domain (1 to 112 aa). Next, because the *pil* locus, including PilG, PilH (112 aa), PilI (178 aa), PilJ (682 aa), PilK (291 aa), and PilL (2472 aa), has been well studied in *P. aeruginosa* (Bertrand et al. 2010), we therefore compared the *pil* locus between *P. aeruginosa* and *L. enzymogenes*. We found that *L. enzymogenes* possessed a homolog of most of the *pil* genes in *P. aeruginosa* (Fig. 1B), including the genes coding for PilG$_{Le}$, PilH$_{Le}$ (94 aa; 73% identity and e value = 3e^{-37}), PilI$_{Le}$ (181 aa; 54% identity and e value = 1e^{-31}), PilJ$_{Le}$ (668 aa; 67% identity and e value = 0), and PilL$_{Le}$ (2398 aa; 71% identity and e value = 0) but no homolog to PilK.

PilG$_{Le}$ positively regulated twitching motility in *L. enzymogenes*. To explore the biological role of *pilG$_{Le}$* in *L. enzymogenes*, we used a genomic marker-free strategy to generate the *pilG$_{Le}$* in-frame deletion mutant (called Δ*pilG*). A 253-bp internal fragment of *pilG$_{Le}$* was deleted in the background of wild-type C3, which was confirmed by PCR and further sequencing. Given that the plasmid-based complementation system does not work in *L. enzymogenes* strain C3 (Kobayashi et al. 2005), we then used a genome integration strategy

to generate a complemented strain of the *pilG$_{Le}$* mutant. For this purpose, the intact *pilG$_{Le}$* gene was inserted into the truncated *pilG$_{Le}$* sequence sites in the background of the *pilG$_{Le}$* mutant using homologous recombination. The complemented strain was verified by PCR and named Δ*pilG*cp in the present study.

Given the role of *pilG* in controlling T4P-mediated cell motility in other bacterial species, we examined whether *pilG$_{Le}$* of *L. enzymogenes* plays a similar role. To test this point, we first evaluated wild-type C3 for twitching motility on the three solid media (water agar, 5% TSA, and 100% TSA) with two different agar concentrations (1.8 and 0.5%). We found that agar concentration and nutrient concentration remarkably influenced twitching motility of wild-type C3 (Fig. 2A). Wild-type C3 exhibited visible twitching motility on water agar and 5% TSA containing 1.8% agar as clear individual or small clusters of cells being separated from the mass of cells at the colony margin. Trails were associated with some of the isolated cells, further evidence that they traveled away from the colony. However, twitching motility was less evident on water agar and 5% TSA when 0.5% agar was used. When the wild-type C3 was grown on 100% TSA, with either 1.8 or 0.5% agar, twitching motility was absent, with no individual cells or cell clusters being separated from the mass of cells at the colony margin.

Next, 5% TSA containing 1.8% agar was used in comparing wild-type C3 and its derivatives for twitching motility. We found that inactivation of *pilG$_{Le}$* abolished wild-type twitching motility, because no individual or small clusters of cells were observed at the margin of the *pilG$_{Le}$* deletion mutant colonies, whereas the complemented strain fully restored wild-type level in this function (Fig. 2B). We also evaluated the colony phenotypes of the wild-type C3 and its derivatives. The wild-type C3 and the *pilG$_{Le}$* complemented strain displayed delicate spreading zones at the periphery of their colonies (Fig. 2B), which was consistent with the gliding phenotype of *L. antibioticus* (Reichenbach 2006). However, no spreading zone around colonies of the *pilG$_{Le}$* deletion mutant colonies was observed under the same condition (Fig. 2B). Taken together, these results indicate that *pilG$_{Le}$* was involved in the positive regulation of twitching motility in *L. enzymogenes*.

Mutation of *pilG$_{Le}$* significantly enhanced HSAF production in *L. enzymogenes* C3. To address whether *pilG$_{Le}$* is involved in the regulation of any secondary metabolite biosynthesis, we determined HSAF production in the *pilG$_{Le}$* mutant in *L. enzymogenes*. To eliminate the potential influence of growth alteration on HSAF production between the wild-type strain and the *pilG$_{Le}$* mutant, we subsequently determined the growth curve (expressed by OD$_{600}$) of wild-type C3, the *pilG$_{Le}$* mutant, and its derivative strains in 10% TSB that was used to cultivate these *Lysobacter* strains for HSAF extraction in this study. We observed that mutation of *pilG$_{Le}$* resulted in a generally similar growth pattern in the logarithmic phase (6 to 16 h) but exhibited a little bit of difference at the time region (18 to 20 h) compared with that of the wild-type strain, where the growth decrease of wild-type C3 was observed (Fig. 3). In detail, mutation of *pilG$_{Le}$* resulted in a slightly delayed growth curve (6 to 18 h) compared with the wild-type C3, whereas the complemented strain partially restored the growth alteration of the *pilG$_{Le}$* mutant to wild-type levels under the same conditions. The results suggested that mutation of *pilG$_{Le}$* had a slight effect on the growth of the wild-type strain in the tested medium (10% TSB).

Next, the antifungal antibiotic HSAF from each tested *Lysobacter* strain cultivated in 10% TSB was extracted and subsequently determined by HPLC. In the present study, to eliminate the potential influence of bacterial growth alteration on HSAF production, we used the indicator of "HSAF production (peak area/OD$_{600}$)" to quantitatively evaluate the ability of HSAF production in *L. enzymogenes*. Here, peak area indicates the areas of HSAF determined by HPLC, whereas the OD$_{600}$ represents the cell density of tested strains at the time point used for HSAF extraction. In this way, we found that inactivation of *pilG$_{Le}$* remarkably increased the yield of HSAF (approximately 2.2-fold), whereas the complemented strain exhibited wild-type HSAF

production levels (Fig. 4A). To verify this point, the transcription of *hsaf pks/nrps* (Lou et al. 2011), the key biosynthetic gene for HSAF production, was quantitatively analyzed between the wild-type C3 and the *pilG_Le* mutant. Based on the results of Figure 3, we finally collected the cells at the logarithmic phase from the wild-type C3, the *pilG_Le* mutant, and the complemented strain at different time points corresponding to the same cell density ($OD_{600} = 1.0$) because, at this cell density, the gene *hsaf pks/nrps* was previously shown to be expressed at the transcriptional level in the wild-type OH11 of *L. enzymogenes* (Qian et al. 2013). The qRT-PCR results showed that mutation of *pilG_Le* significantly enhanced the transcription of *hsaf pks/nrps* (approximately 2.7-fold) (Fig. 4B), which was consistent with the result of HSAF yield in the *pilG_Le* mutant. This indicated that the contribution of *pilG_Le* to HSAF biosynthesis was, at least partially, through enhancing the transcription of *pks/nrps* in *L. enzymogenes*. Collectively, these results demonstrated that *pilG_Le* was involved in the negative regulation of HSAF production in *L. enzymogenes*.

DISCUSSION

In this study, we found that *pilG_Le* in *L. enzymogenes* C3 upregulates twitching motility while downregulating HSAF production, providing evidence that *pilG_Le* in *L. enzymogenes* has opposite effects on different pathways relating to antagonism and biological control against fungi. Although we did not examine the production or functioning (extension and retraction) of T4P

directly in this study, T4P has been reported to be involved in twitching motility of two strains (C3 and OH11) of *L. enzymogenes* (Patel et al. 2011; Wang et al. 2014). The collective results support the supposition that *pilG_Le* in *L. enzymogenes* C3 affects twitching motility by positively regulating TP4 biosynthesis or function in ways akin to those reported in *P. aeruginosa*.

In this study, we found that PilG_Le in *L. enzymogenes*, on the basis of its amino acid sequence, is a response regulator (RR) which typically functions in bacteria as a component of phosphotransfer signal transduction pathways commonly referred to as two-component transduction systems (Stock et al. 2000). PilG_Le is a single domain (SD)-RR (Jenal and Galperin 2009), because it contains a REC domain but lacks a dedicated output domain. Importantly, the presence of a conserved REC domain in PilG_Le suggests that this SD-RR is probably involved in receiving a signal from an unknown sensor protein via a phosphotransfer mechanism. This hypothesis is further supported by the finding of existence of three predicted phosphorylation active sites (Asp-5, Asp-48, and Lys-100) in PilG_Le that are highly conserved among diverse bacteria such as *Salmonella typhimurium* and *P. aeruginosa* (Darzins 1993). The hypothetical sensor protein in *L. enzymogenes* is worthy of investigation because it could provide clues to the environmental cues that trigger antibiotic production and T4P-related activities in this bacterial species.

In *P. aeruginosa*, PilG appears to regulate twitching motility via two mechanisms. In one, phosphorylated forms of PilG and PilH, an RR located adjacent to PilG, interact with the T4P motor ATPases
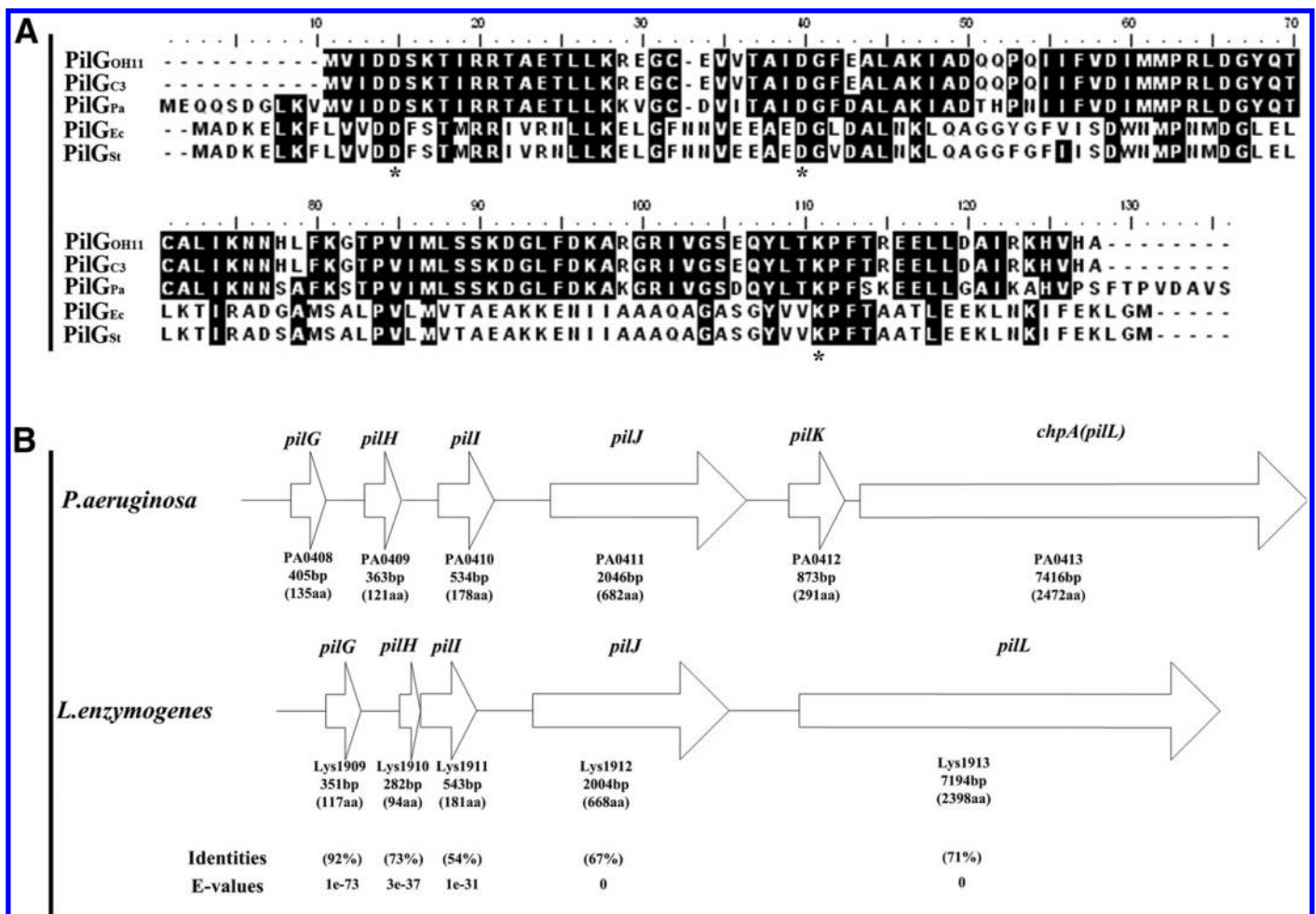

Fig. 1. Identification of PilG_Le in *Lysobacter enzymogenes*. **A,** Sequence alignment of PilG_Le in *L. enzymogenes* strains OH11 and C3 (PilG_OH11 and PilG_C3, respectively) with other PilG proteins, including PilG_Ec (GI: 190906748) of *Escherichia coli*, PilG_Pa (GI: 15595605) of *Pseudomonas aeruginosa*, and PilG_st (GI: 153909) of *Salmonella typhimurium*; an asterisk (*) indicates the three predicted phosphorylation active sites. **B,** Comparison of the *pilG_Le* locus between *L. enzymogenes* strain OH11 and *P. aeruginosa*. In comparison with the counterpart in *P. aeruginosa*, the identity and e value of each protein homolog in *L. enzymogenes* was shown. The identify card (ID) number and size at both the nuclear-acid and amino-acid level of each gene was provided in this figure.

PilB and PilT to extend and retract T4P, respectively (Bertrand et al. 2010). It is likely that $PilG_{Le}$ regulates T4P extension in a similar manner because homologs to the T4P motor complex genes reported in *P. aeruginosa*, including *pilB*, are found in the *L. enzymogenes* OH11 genome (Supplementary Fig. S3). In the other twitching motility regulatory pathway in *P. aeruginosa,* PilG regulates biogenesis of T4P by controlling intercellular levels of cAMP (Fulcher et al. 2010). In this case, cAMP binds the cAMP-receptor protein Vfr, and then the cAMP-Vfr complex regulates T4P biogenesis (Whitchurch et al. 2005). *L. enzymogenes* has a Vfr homolog (namely, Clp) that shares 69% similarity to Vfr at the amino-acid sequence level (Kobayashi et al. 2005; Wang et al. 2014). We previously showed that Clp regulation of twitching motility in *L. enzymogenes* was through the modulation of T4P (Wang et al. 2014). This raises the possibility that $PilG_{Le}$ affects T4P biosynthesis by interacting with Clp. However, Clp proteins from *Xanthomonas campestris* pv. *campestris* (*Xcc*) and *Stenotrophomonas maltophilia* (*Sm*), which are in the same bacterial family as *L. enzymogenes*, do not bind cAMP but, instead, act as receptors to c-di-GMP (Tao et al. 2010). Furthermore, Clp of *L. enzymogenes* OH11 shares with Clp_{Xcc} and Clp_{sm} a conserved amino acid residue, E99, at the N terminus, which is a signature binding site with c-di-GMP (Tao et al. 2010; Wang et al. 2014).

Genome scanning revealed that *L. enzymogenes* OH11 contained a total of 26 proteins containing GGDEF, EAL, and HD-GYP domains (data not shown). The GGDEF-containing proteins are characterized as diguanylate cyclases to synthesize c-di-GMP by using 2 GTP as substrates, while the EAL and HD-GYP domain-containing proteins were defined as phosphodiesterases responsible for degradation of c-di-GMP into pGpG and 2 GMP, respectively (Tal et al. 1998; Galperin et al. 1999). These data suggest that the intracellular c-di-GMP concentration may be modulated by these domain-containing proteins in response to different environmental stimuli in *L. enzymogenes*. Although we currently lack direct evidence for the involvement of intercellular c-di-GMP in T4P-dependent twitching motility in *L. enzymogenes*, a number of studies have demonstrated intercellular c-di-GMP levels to play important roles in modulating cell motility in other bacterial species (Römling et al. 2013). Therefore, if $PilG_{Le}$ interacts with Clp to regulate T4P-dependent twitching motility in *L. enzymogenes*, it would likely involve c-di-GMP rather that cAMP as the intermediate signal, forming a signal cascade that would have considerable differences from the well-characterized PilG/cAMP/Vfr cascade in *P. aeruginosa* (Fulcher et al. 2010; Whitchurch et al. 2005).

A relationship between DSF and PilG signaling in the regulation of twitching motility is currently unknown in *L. enzymogenes*. However, disruption of DSF system enhanced twitching motility in the nutrient-rich medium nutrient yeast glycerol, whereas the wild-type strain almost lost this ability under the same testing conditions (Qian et al. 2013), suggesting that DSF negatively regulated twitching motility under nutrient-rich conditions. In contrast to this finding, we found both the wild-type OH11 and the DSF-deficient mutant $\Delta rpfF$ (Qian et al. 2013) exhibited a clear twitching motility on the 5% TSB agar, the nutrient-limited medium described in this study (Supplementary Fig. S4). This result suggests that *L. enzymogenes* DSF signaling exerts its regulatory effect on twitching motility, most probably, under the nutrient-rich conditions. Such a regulatory type of DSF signaling on twitching motility was remarkably different from the PilG, signaling, because mutation of *pilG* completely abolished twitching motility on 5% TSB agar. Collectively, these suggest that DSF and PilG signaling pathways may act under different conditions to regulate twitching motility independently in *L. enzymogenes*.

The various functions previously reported to be regulated by PilG have been limited to those related to involving T4P (e.g., twitching
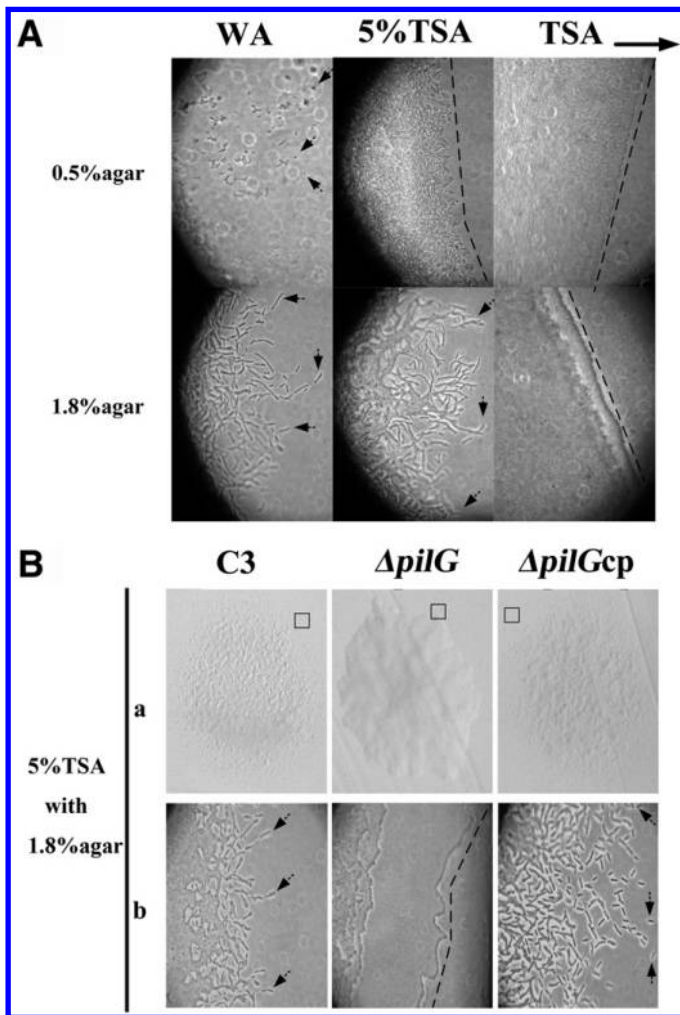

Fig. 2. Twitching motility in *Lysobacter enzymogenes* strain C3 **A,** Twitching motility of the wild-type strain on water agar (WA), 5% tryptic soy agar (TSA) and 100% TSA containing 0.5 or 1.8% agar. **B, a,** Colony phenotype and **b,** twitching motility of various *Lysobacter* strains. The direction of the solid arrow on the top right corner indicates the direction of cell movement. Cells indicated by the dashed arrow are the representative mobile cells from the insert of part "a". The dashed line indicated the colony edge of the twitching-motility-deficient mutant. $\Delta pilG$ = the $pilG_{Le}$ deletion mutant and $\Delta pilG$cp = the genomic integrated complemented strain of the $pilG_{Le}$ mutant.
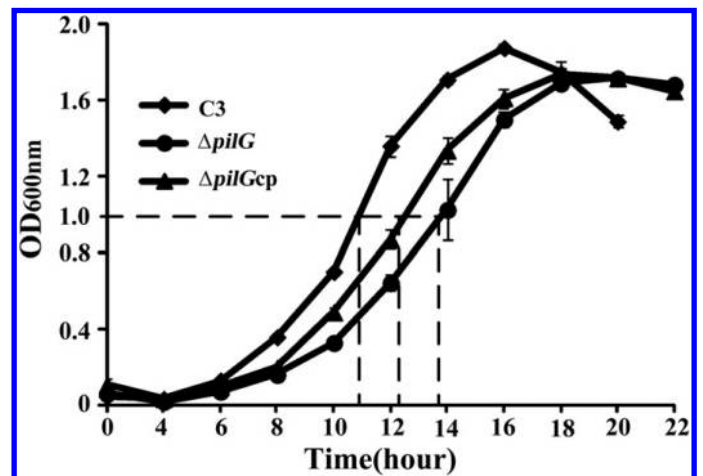

Fig. 3. Growth curves of various *Lysobacter* strains in 10% tryptic soy broth medium. The growth level of each strain was measured by the optical density at 600 nm (OD_{600}) at regular intervals (2 or 4 h). Three replicates for each treatment/strain were used, and the experiment was performed three times. Vertical bars represent standard errors. The dashed line indicates that the cells at the logarithmic phase from the wild-type C3, the $pilG_{Le}$ mutant ($\Delta pilG$), and the complemented strain ($\Delta pilG$cp) at different time points correspond to the same cell density (OD_{600} = 1.0) and were collected for RNA extraction.

motility, attachment, and DNA uptake) (Bertrand et al. 2010; Tønjum et al. 1995; Whitchurch et al. 2004; Yoshihara et al. 2002). Thus, our finding in this study that $PilG_{Le}$ plays a role in the regulation of HSAF biosynthesis production in *L. enzymogenes* represents, to our knowledge, the first demonstration of PilG regulation of secondary-metabolite antibiotic biosynthesis in a bacterial species. Although the mechanism for $PilG_{Le}$ regulation of HSAF production is currently unknown, it most probably is not associated with Clp signaling because mutation of *clp* and *lat,* which encodes an acetyltransferase regulated by *clp*, almost abolished HSAF production (Wang et al. 2014), whereas mutation of $pilG_{Le}$ enhanced HSAF production in this study. Furthermore, our finding that $PilG_{Le}$ regulates HSAF production and twitching motility in opposite directions implies that distinct signaling pathways downstream from $PilG_{Le}$ lead to expression of the two traits. Interestingly, the DSF quorum-sensing signaling in *L. enzymogenes* appears to regulate HSAF production and cell motility in opposite directions from $PilG_{Le}$ (Qian et al. 2013). We speculate that each signaling system acts under different conditions to activate or repress HSAF biosynthesis independently. To provide evidence to support this hypothesis, we added a DSF molecule called DSF3 produced by *L. enzymogenes* into the $pilG_{Le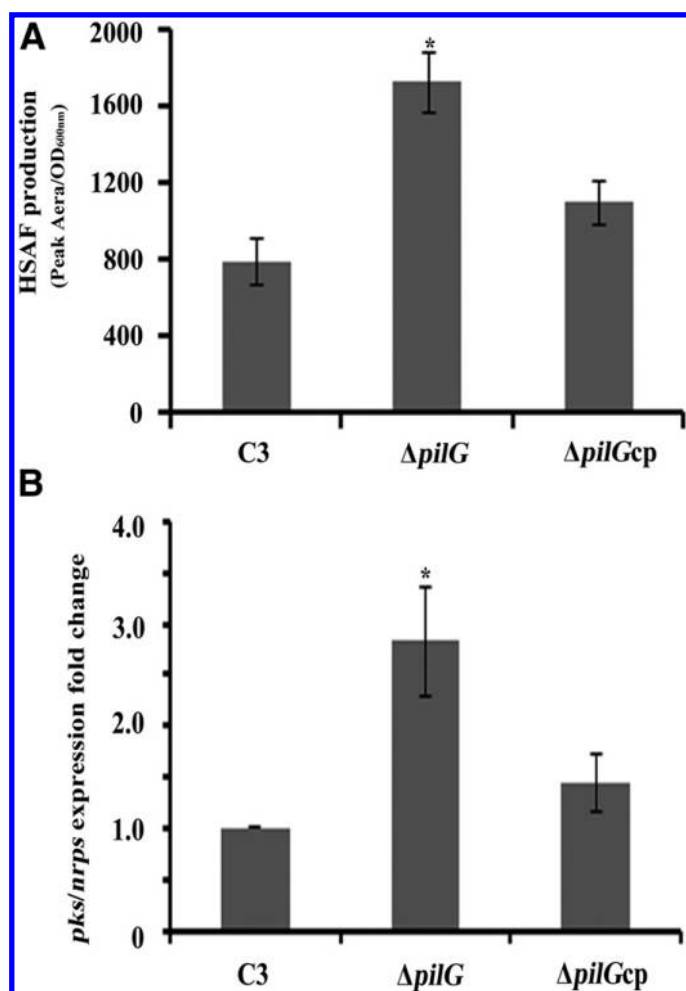}$ mutant to test its effect on HSAF production. DSF3 was previously shown to induce HSAF biosynthesis via the RpfC-RpfG two-component transduction system and the regulator Clp in *L. enzymogenes* (Han et al. 2015). We found that the DSF3-treated *pilG* mutant produced higher HSAF level than the DSF-untreated strain (the *PilG* mutant treated by the solvent), indicating that the induction of DSF3 on HSAF production was not disrupted when missing *pilG* in *L. enzymogenes* (Supplementary Fig. S5). This point further suggests that the DSF signaling pathway regulated HSAF biosynthesis via a *pilG*-independent way in *L. enzymogenes*. Native production of DSF3 being extremely low in *L. enzymogenes* makes it difficult to detect the molecule by HPLC (Han et al. 2015). In this case, we did not determine the DSF3 level in the *pilG* mutant at this moment. Therefore, whether *pilG* has an effect on DSF3 production needs to be further investigated in future studies. Nevertheless, all these points allow us to speculate that DSF and PilG signaling act under different conditions to modulate twitching motility and HSAF biosynthesis independently. Populations of *L. enzymogenes* in nature likely switch back and forth from a motile state to a static state depending on population density and substrate availability. When in low population numbers or under nutrient-deficiency conditions, $PilG_{Le}$ might activate twitching motility as a mechanism for movement to organic compounds for saprotrophic growth or to fungal hyphae for parasitic growth. Because the biosynthesis of secondary metabolites requires energy, it follows that production of compounds such as HSAF is downregulated by $PilG_{Le}$ during the motile phase. Upon reaching a suitable substrate or adhering to a fungal host, the population increases to the point in which the DSF quorum-sensing system inactivates motility and initiates biosynthesis of antibiotics such as HSAF to compete against other microorganisms for the substrate or to suppress growth of the fungal host. Testing this hypothesis will make for intriguing future research whose results could lead to better understanding of the functions of biocontrol-related traits in nature and, potentially, to more effective utilization of bacteria such as *L. enzymogenes* as biological control agents.

Fig. 4. Mutation of $pilG_{Le}$ significantly increased the production of a heat-stable antifungal factor (HSAF) in *Lysobacter enzymogenes*. **A,** Quantitative measurement of the yield of HSAF in the wild-type strain and its derivative strains. **B,** Quantitative determination of the transcription of the critical biosynthetic gene (*hsaf pks/nrps*) for HSAF. Each column indicates the mean of three biologically independent experiments. Vertical bars represent standard errors; an asterisk (*) ($P < 0.05$; *t* test) above the bars indicates a significant difference between the wild-type strain and the $pilG_{Le}$ mutant. C3 = the wild-type strain of *L. enzymogenes*, $\Delta pilG$ = the $pilG_{Le}$ deletion mutant, and $\Delta pilG$cp = the genomic integrated complemented strain of the $pilG_{Le}$ mutant.

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Table S1 Primers used for in-frame deletion, complementation and qRT-PCR in this study

Primer	Sequences (5'-3')[a]	Purpose	Resource
1909F-1	GG<u>GGTACC</u>CAATTCGTGCCAGCCGTTCT (*Kpn*I)	To amplify a 656-bp upstream homologue arm of *pilG*	This study
1909F-2	CCC<u>AAGCTT</u>TCGCATCCCTCGCGTTTC (*Hin*dIII)		
1909R-1	CCC<u>AAGCTT</u>CTCGACGCGATCCGCAAGCA (*Hin*dIII)	To amplify a 597-bp downstream homologue arm of *pilG*	This study
1909R-2	GC<u>TCTAGA</u>CAGCGACAGGATTTCCACCA (*Xba*I)		
C1	CTGCCGAGTTCGATCTGTGA	To verify the *pilG* deletion mutant.	This study
C2	CGCCTTCCAGGAACTGCTT		
C1909-F	CG<u>GGATCC</u>GCTTGCGTATTCAGTTTCCG (*Kpn*I)	To amplify a 570-bp DNA fragment containing the intact *pilG* gene and its predicted promoter	This study
C1909-R	CCC<u>AAGCTT</u>CGCAGAGTCCTCGATAAGCAG (*Hin*dIII)		
C1909YZ-F	CCTCTTCGCTATTACGCCAG	To verify the construction of the genomic integrated complemented strain of the *pilG* mutant. An expected PCR size from this complemented strain is approximate 870 bp	
C1909YZ-R	ATCCGCTTGGTCTGCTCGTCCC		
16S rRNA-1	ACGGTCGCAAGACTGAAACT	The housekeeping gene for real-time quantitative PCR	Qian et al., 2013
16S rRNA-2	AAGGCACCAATCCATCTCTG		
pks/nrps-1	ACTATTTGTTGGGCGACGAC	To determine the transcriptional level of *pks/nrps*	Qian et al., 2013
Pks/nrps-2	GTAACCGAACAGGGTGCAAT		

[a] Restricted digestion enzyme site was underlined.
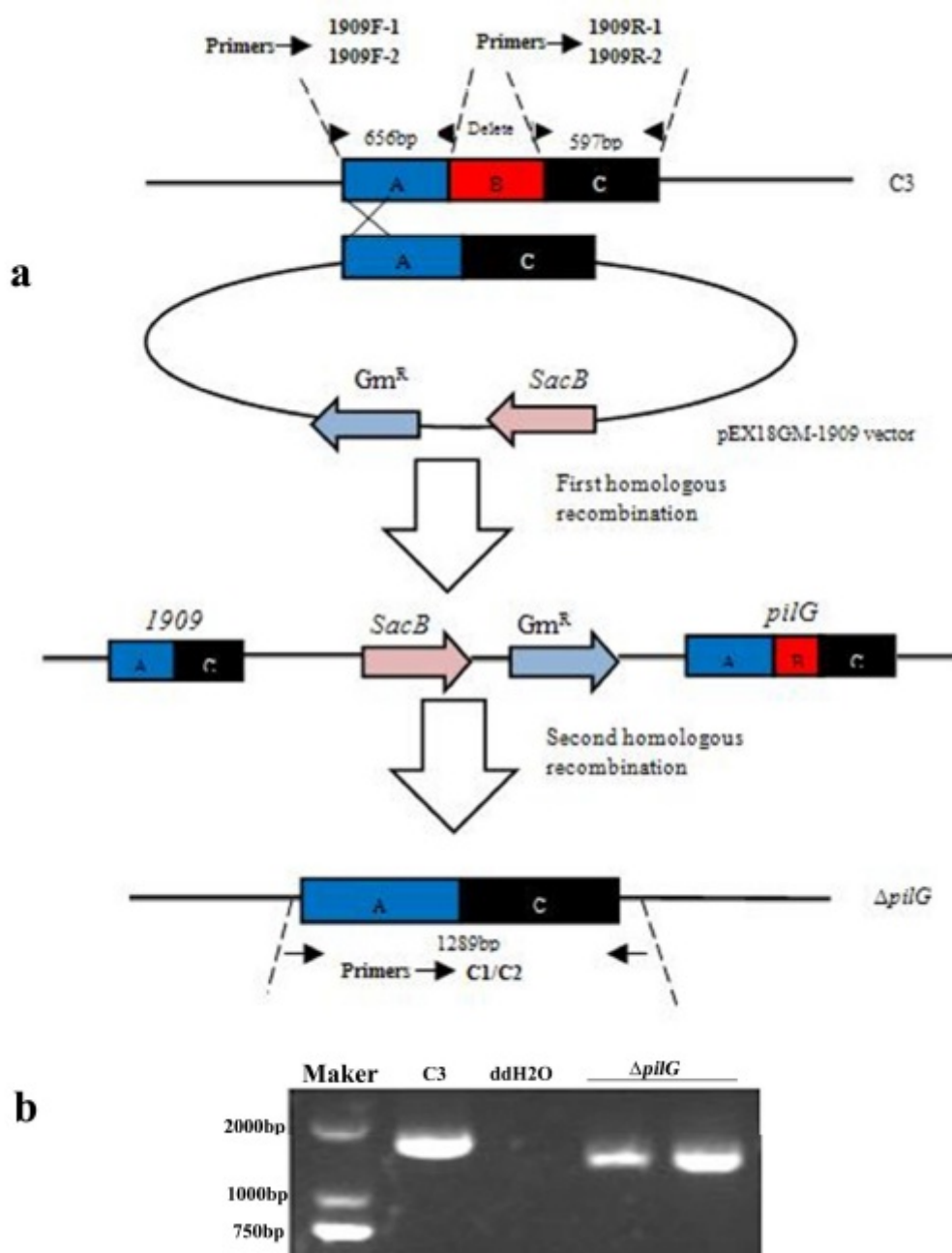
Supporting Result

Fig. S1 Construction and confirmation of the *pilG$_{Le}$* mutant in *Lysobacter enzymogenes*. (A) The scheme of gene deletion was shown. The 656-bp (amplified by 1909F-1/1909F-2) (Table S1) and 597-bp (amplified by 1909R-1/1909R-2) (Table S1) DNA fragment was used as 5' and 3' homolouge arm for double crossover recombination, respectively. The internal 253-bp DNA fragment would be deleted in the *pilG$_{Le}$* mutant. The primers C1/C2 (Table S1) was used for molecular

confirmation of $pilG_{Le}$ mutant. If the 253-bp internal fragment of $pilG_{Le}$ was successfully deleted, a 1289-bp DNA fragment would be amplified from the $pilG_{Le}$ mutant. (B) PCR confirmation of the $pilG_{Le}$ mutant. Due to deletion of 253-bp internal fragment of $pilG_{Le}$, a ~1.2-kb DNA fragment was amplified from the $pilG_{Le}$-deletion mutant. C3, the wild-type strain of *L. enzymogenes*; Δ*pilG*, the $pilG_{Le}$ deletion mutant; ddH2O, a negative control. This gene-deletion strategy was used for construction of other gene mutants described in the present study.
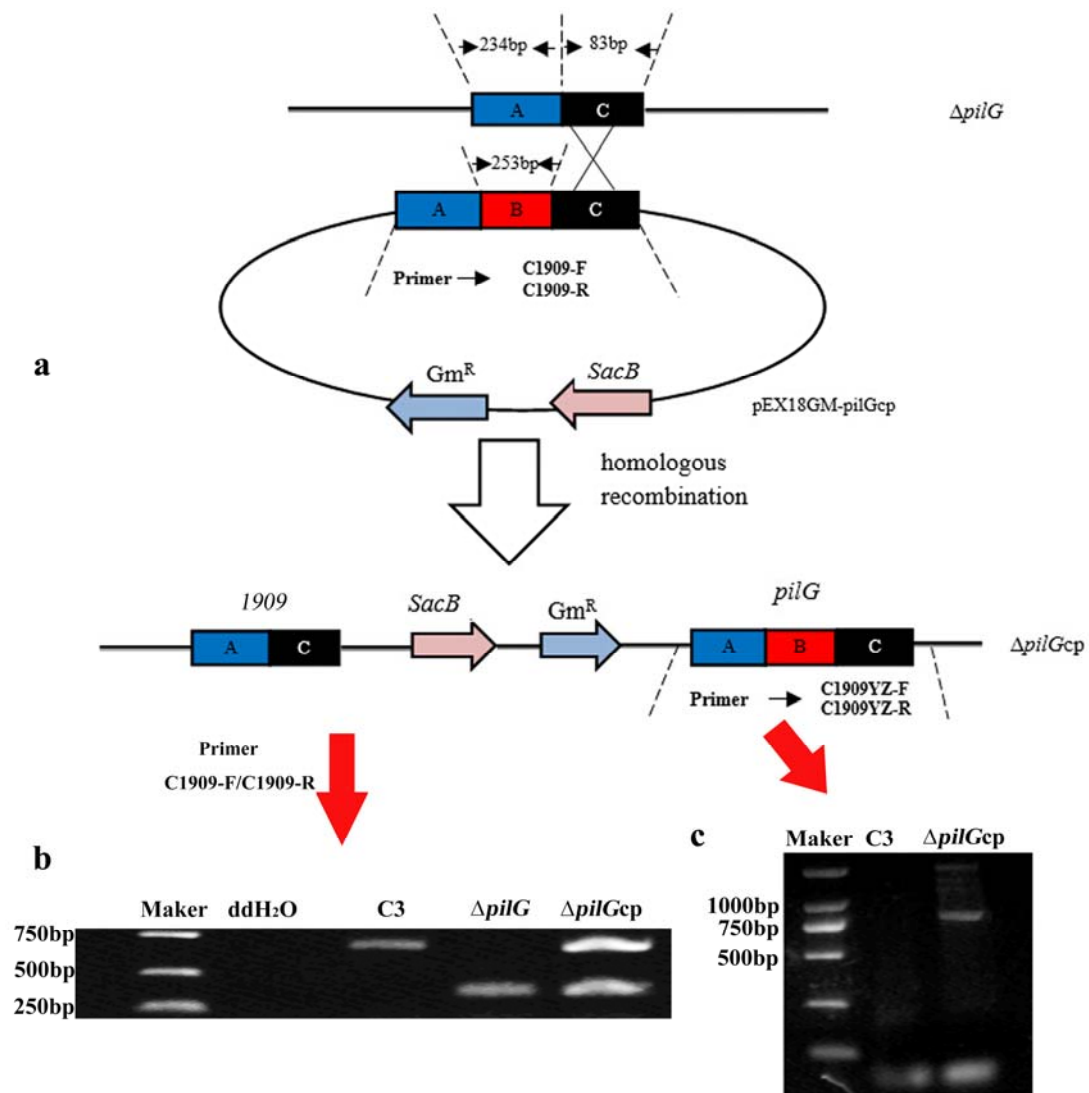
Fig. S2 Construction and verification of the genomic integrated complementary strain of the *pilG_Le* mutant in *Lysobacter enzymogenes*. (A) The scheme for constructing the genomic integrated complementary strain of the *pilG_Le* mutant was shown. A 570-bp (amplified by C1909-F/C1909-R) (Table S1) DNA fragment, including *pilG_Le* and its predicted promoter, was used as the homologue arm for single-crossover recombination. After corrected insertion, due to the insert of an intact *pilG_Le* (570 bp) into the truncated *pilG_Le* (317 bp) in the background of the *pilG_Le* mutant, the expected complemented strain should both possessed the truncated and intact *pilG_Le*. Thus, two DNA fragments correspond to the truncated and intact *pilG_Le* should be amplified from the corrected *pilG_Le* complemented strain, which was

subsequently confirmed by PCR (B). To further confirm the correction of the *pilG*_{*Le*}

complemented strain, a pair of primers (C1909YZ-F/R) was designed (Table S1). One

primer (C1909YZ-R) was designed based on the sequence of 3' of *pilG*_{*Le*}, whereas the

other (C1909YZ-F) was designed on the basis of the sequence of the inserted vector.

Thus, the expected DNA fragment should be only amplified from the complemented

strain using this pair of primers, while the amplified signal from the *pilG*_{*Le*} mutant

should be negative, as no paired sequence corresponds to the primer C1909YZ-F was

existed in the *pilG*_{*Le*} mutant, which was experimentally verified by PCR (C).
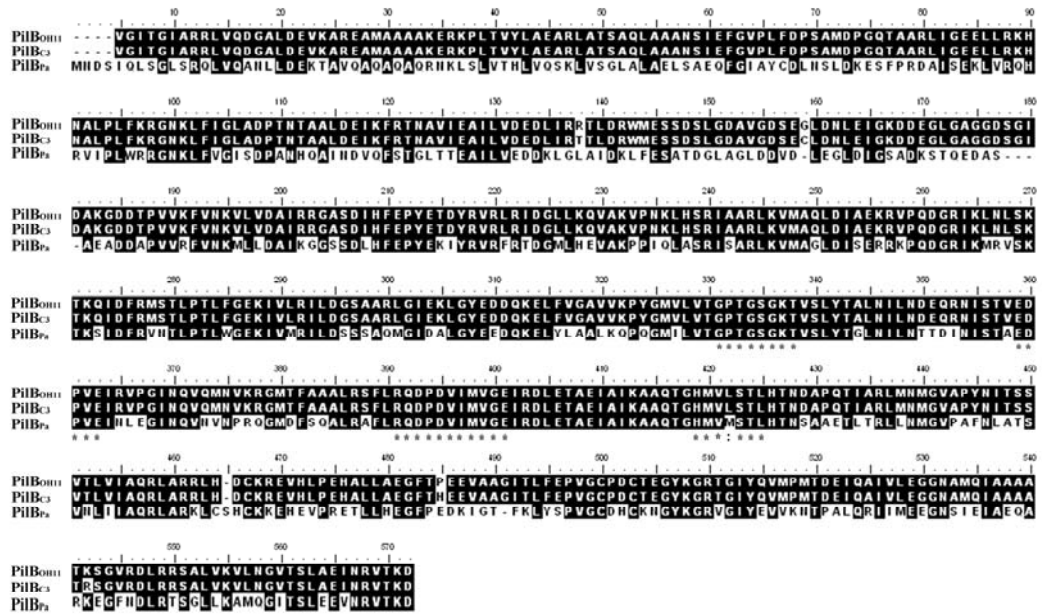
Fig. S3 Sequence alignment of PilB of *L. enzymogenes* with the counterpart PilB (ID: GI:15599722)) from *Pseudomonas aeruginosa*.
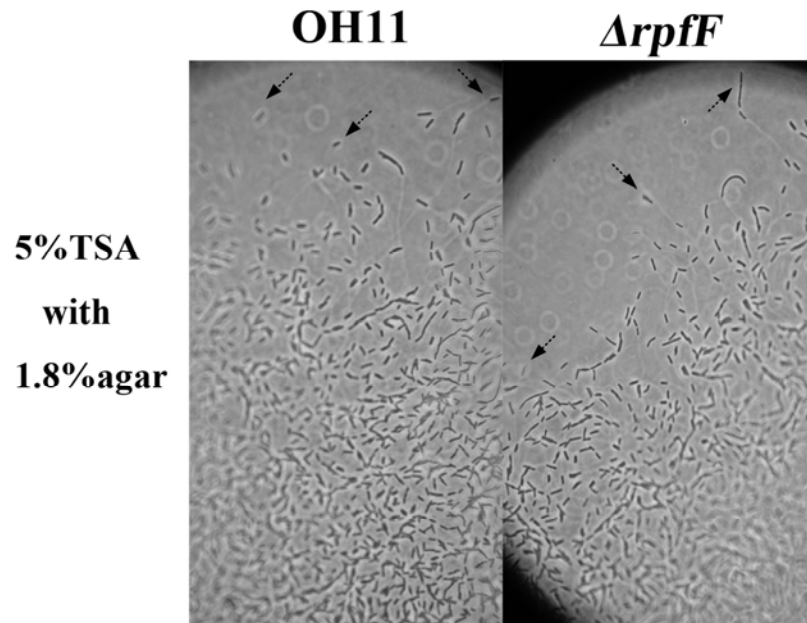
Figure S4. The effect of DSF signaling on twitching motility of *L. enzymogenes* on nutrient-limiting medium. Disruption of DSF signaling did not impair cell twitching motility in L. enzymogenes OH11. The cells indicated by the dashed arrow are the representative mobile cells. OH11, the wild-type strain; Δ*rpfF*, the DSF-disrupted strain (Qian et al., 2013).
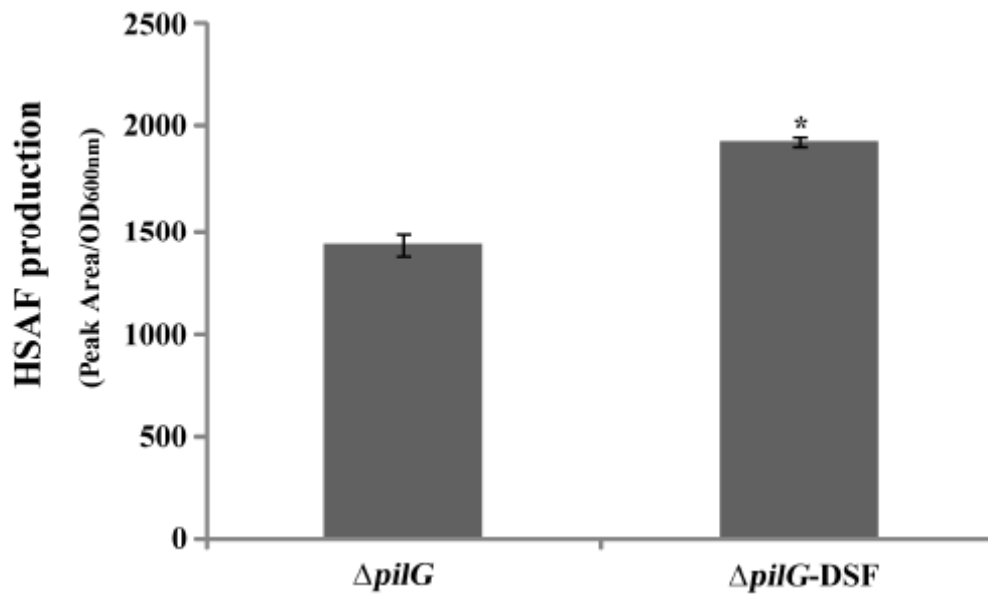
Figure S5. The effect of *L. enzymogenes* DSF3 on HSAF production in the background of *pilG*$_{Le}$ muation. $\Delta pilG$, the *pilG*$_{Le}$ deletion mutant treated by the solvent (ethanol); $\Delta pilG$-DSF, the *pilG*$_{Le}$ deletion mutant treated by DSF3, which is a Diffusible Signal Factor produced by *L. enzymogenes* (Han et al., 2015). The experiments were performed three times and each involves three replicates. Vertical bars represent standard errors. '*' (p<0.05; t-test) above the bars indicate a significant difference between the DSF3 treatment and the control in the *pilG*$_{Le}$ mutant on HSAF production.