A non-flagellated biocontrol bacterium employs a PilZ-PilB complex to provoke twitching motility associated with its predation behavior

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Abstract

Lysobacter enzymogenes OH11 is a non-flagellated, ubiquitous soil bacterium with broad-spectrum antifungal activities. Although lacking flagella, it employs another type of motile behavior, known as twitching motility that is powered by type IV pilus (T4P) to move towards neighboring crop fungal pathogens to kill them as food. At present, little is known about how this non-flagellated bacterium controls twitching motility that is crucial for its predatory lifestyle. Herein, we present a report on how a non-canonical PilZ domain, PilZ<sub>Le3639</sub>, controls such motility in the non-flagellated L. enzymogenes; it failed to bind with c-di-GMP but seemed to be required for twitching motility. Using bacterial two-hybrid and pull-down approaches, we identified PilB<sub>Le0708</sub>, one of the PilZ<sub>Le3639</sub>-binding proteins that are essential for the bacterial twitching motility, could serve as an ATPase to supply energy for T4P extension. Through site-mutagenesis approaches, we identified one essential residue of PilZ<sub>Le3639</sub> that is required for its binding affinity with PilB<sub>Le0708</sub> and its regulatory function. Besides, two critical residues within the ATPase catalytic domains of PilB<sub>Le0708</sub> were detected to be essential for regulating twitching behavior but not involved in binding with PilZ<sub>Le3639</sub>. Overall, we illustrated that the PilZ-PilB complex formation is indispensable for twitching motility in a non-flagellated bacterium.

Keywords: Twitching motility, PilZ, PilB, Lysobacter, Type IV pilus

Background

The Gram-negative genus of Lysobacter comprises more than 30 species that are emerging as important sources of crop biocontrol agents. They are novel because they exhibit proficient abilities to generate a wide variety of anti-infectious metabolites and extracellular lytic enzymes (Christensen and Cook 1978; Kobayashi et al. 2005; Xie et al. 2012; Panthee et al. 2016). Recent comparative genomic studies uncovered that most members of Lysobacter do not carry a FliC homolog, which encodes flagellin required for the biogenesis of surface-attached flagella (de Brujin et al. 2015). Therefore, with minor exceptions, most Lysobacter species are, although ubiquitous in the environment, non-flagellated (Christensen and Cook 1978; Hayward et al. 2010).

How do the Lysobacter species without flagella migrate towards a more favorable environment or escape from undesirable conditions in their natural niches? Our earlier studies revealed that surfaced-attached type IV pilus (T4P) can power Lysobacter species to move in a twitching mode (Zhou et al. 2015). This motile behavior seems to facilitate the non-flagellated Lysobacter species to draw near ecologically-relevant pathogens. When Lysobacter species establish the contact with pathogens, they

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predate or kill them, hence protect the plants from being infected (Xia et al. 2018).

*P. aeruginosa* is a plant-associated, soil proteobacterium that acts as a predator of crop fungal pathogens (Qian et al. 2009). To prey on nearby fungi, *P. aeruginosa* is required to move towards them via T4P-dependent twitching motility and further kills these microorganisms through secreting an antibiotic, known as heat-stable antifungal factor, HSAS (Yu et al. 2007; Xia et al. 2018). In our earlier studies, we demonstrated 19 pilus structural or regulatory component proteins, including the major pilus subunit, PilA and the motor proteins PilB and PilT, are required for the biogenesis of T4P and the function of twitching motility in *P. aeruginosa* OH11 (Xia et al. 2018).

Genes encoding proteins with PilZ domains are widely distributed in a variety of bacterial genomes and control numerous cellular processes, such as biofilm formation, T4P biogenesis, twitching motility, two-component signal transduction, and virulence (Galperin and Chou 2020). These domains could be stand-alone, in tandem, or fused with other signaling domains, such as EAL, GGDEF, or HD-GYP that are highly associated with the signaling of c-di-GMP, a ubiquitous bacterial second messenger. Furthermore, proteins with PilZ domains are widely distributed in both flagellated and non-flagellated bacterial species. PilZ domains are highly conserved and provide further insights on the functional and mechanistic diversity of PilZ domains in bacteria.

**Results**

**PilZLe3639 is a stand-alone PilZ domain unable to bind c-di-GMP**

A genomic search of strain OH11 led us to discover Le3639, a homologous PilZ protein to PilZP_A2960 protein that is referred to as PilZLe3639 herein. PilZLe3639 shares a very high (76–83%) sequence similarity with PilZP_A2960 and contains no conserved c-di-GMP-binding signature motifs, as described above (Fig. 1a). The objective of this work is to determine whether PilZLe3639 can affect the twitching motility of the non-flagellated *P. aeruginosa*, and if yes, what is the underlying mechanism? Herein, we showed that PilZLe3639 is indeed involved in controlling the twitching motility of *P. aeruginosa*. Moreover, PilZLe3639 seems to use the same residue critical for its role in twitching motility and interaction with PilB_L0708. We underscore for the first time the involvement of the PilZ-PilB protein complex in twitching motility in non-flagellated bacteria, which provides further insights on the functional and mechanistic diversity of PilZ domains in bacteria.
ΔPilZLe3639, an in-frame Lysobacter deletion mutant (Additional file 1: Table S1) via a double homologous-recombination approach as described earlier (Qian et al. 2012). In the twitching-motility inducing medium (1/10 TSA), we found that the wild-type OH11 displayed an apparent twitching behavior by spreading single or clustered cells outside from the colony margin (Fig. 2). At the same time, the inactivation of PilZ Le3639 completely abolished this twitching phenomenon (Fig. 2). The twitching motility defect in the PilZLe3639 mutant was...
fully rescued by complementation with a plasmid-borne PilZLe3639 (Fig. 2) but not by an empty plasmid (Fig. 2). These results imply the crucial role of PilZ Le3639 in trig-

gering the Lysobacter twitching motility. According to an earlier study (Guzzo et al. 2009), Tyr24 (Y24) and Trp71 (W71) are the two potential key residues for exe-

cuting the biological function of PilZ Xac1133. To figure out whether they also play a similar role in PilZ Le3639, we generated two individual mutants of Y24A (A is an abbreviation of Alanine) and W71A, respectively, and complemented these PilZ Le3639 variants with the plasmid harboring Y24A or W71A substitution. The results (Fig. 2) showed that the Y24 mutant behaves like the wild-
type allele and can rescue the twitching motility defect of the pilZ Le3639 mutant. However, the W71 mutant allele did not restore the normal twitching motility phenotype, indicating W71 is a key residue for protein activity. These results collectively suggest that PilZ Le3639 is required for twitching motility, and the amino residue of W71 is essential for displaying such a function.

Binding of PilZ Le3639 with PilB Le0708 is required for twitching motility

To understand how PilZ Le3639 modulates the Lysobacter twitching motility, we screened a total of 19 pilus-associated structural or regulatory proteins (Xia et al. 2009).
to identify the direct PilZLe3639-binding partner(s) (Fig. 3a). For screening, we employed the bacterial adenylate cyclase two-hybrid (BACTH) system (Ouellette et al. 2017). Each of the 19 genes was individually cloned into the pKT25 vector to generate the prey pKT25 constructs. Along with the bait plasmid pUT18c-PilZLe3639, each of the recombinant prey pKT25 constructs was co-transformed into the E. coli BTH101, which was further grown in a selective medium. Based on the principle of the BACTH system, if bait PilZLe3639 established a direct interaction with the prey protein, the co-transformed E. coli strain would exhibit a “blue” phenotype. Via this approach, we found that among the 19 proteins, only PilBLe0708 interacted directly with PilZLe3639 (Fig. 3b). To validate this observation, we further tested the ability of PilZLe3639-His to pull down GST-PilBLe0708, which was indeed the case since we observed a positive signal (Fig. 3c). These results reveal that PilZLe3639 directly interacted with PilBLe0708 of L. enzymogenes.

Multiple sequence alignment showed that PilBLe0708 possessed two conserved motifs, the Walker A and Walker B (boxed in red rectangular in Fig. 4a), both of which are highly required for its enzymatic activity (Chiang et al. 2008). Between the predicted Walker A and B motifs, the residues of Lys333 (K333) and Glu397 (E397) of PilB Xac3239 were previously shown to play potential key roles in forming the PilB Xac3239-PilZ Xac1133 complex (Guzzo et al. 2009). Therefore, we constructed the PilBLe0708 variants with a K333A or E397A substitution in the wild-type chromosome and subsequently tested the ability of the mutant strains in producing the L. enzymogenes twitching motility. We found that the chromosomal replacement of Lys333 or Glu397 in the wild-type OH11 completely abolished the bacterial twitching motility (Fig. 4b), suggesting that both residues are essential for the function of PilBLe0708, which supports the earlier notion that the enzymatic activity and functionality of PilB are highly associated. To validate that the above observations are site specific, two unrelated residues, Pro208 or Tyr209 of PilBLe0708, were further selected for replacement by Alanine (A) (Fig. 4a). We found that the P208A or Y209A substitution did not seem to affect the L. enzymogenes twitching motility (Fig. 4b). Therefore, the PilZLe3639-PilBLe0708 binding pair appears to correlate well with their functional outcomes in regulating the formation of twitching motility in L. enzymogenes.

W71 of PilZLe3639 is required for PilZLe3639-PilBLe0708 interaction

To provide molecular details for the PilZLe3639-PilBLe0708 binding, we tried to identify key residue(s) of PilZLe3639 or PilBLe0708 that are required for their interaction. Finally, a GST pull-down assay confirmed the direct binding of PilZLe3639 with PilBLe0708 in vitro.

Fig. 3 PilZLe3639 directly interacted with PilBLe0708, a characterized pilus motor protein. a, b PKT25-PilZLe3639 with the recombinant pUT18c carrying different pilus-associated proteins that are required for twitching motility in L. enzymogenes OH11 (Xia et al. 2018) were co-transformed into the E. coli BTH101 cells to test their potential protein-protein interactions by the bacterial adenylate cyclase two-hybrid (BACTH) system. The co-transformed E. coli colony with a “blue” color indicated the test protein-protein interactions happened under the test conditions. Using this approach, only PilBLe0708 with PilZLe3639 showed an interaction signal. ‘+’, a positive control (pUT18c-Zip & pKT25-Zip), ‘-’, a negative control (pUT18c & pKT25). c GST pull-down assay confirmed the direct binding of PilZLe3639 with PilBLe0708 in vitro.
that are important for their binding and function. For this purpose, we first selected the residues of W71 and Y24 of PilZ Le3639 as a comparable pair, because we already showed that the former was necessary for the involvement of PilZ Le3639 in twitching motility, while the latter was not (Fig. 2). Using the BACTH system, we found that the PilZ Le3639 W71A variant exhibited almost entirely impaired binding capability, while the PilZ Le3639 Y24A variant did not seem to demonstrate any binding with PilB Le0708 (Fig. 5a). These results collectively indicate that W71 is a key residue of PilZ Le3639 in determining its binding affinity with PilB Le0708 and to regulate the in vivo twitching motility.

To map the residues of PilB Le0708 that are essential for influencing its direct interaction with PilZ Le3639, we tested the binding of the PilZ Le3639 with several PilB Le0708 variants (PilB Le0708 K333A, PilB Le0708 E337A, PilB Le0708 P208A, and PilB Le0708 Y209A) through the BACTH system and found that, like the native PilB Le0708, all the tested PilB Le0708 variants seemed to be able to bind well with PilZ Le3639 under the testing conditions (Fig. 5b). These results suggest that while K333 and E397 of PilB Le0708 that are important for their binding and function.
PilBLe0708 and PilZLe3639 are required for its own regulation in twitching motility, both of them are not likely involved in the PilBLe0708-PilZLe3639 binding.

Discussion
Twitching motility is arguably one of the best-characterized motile behaviors in bacteria (Burrows 2012). Unlike the flagellum-driven swimming or swarming motility, the formation of twitching motility is powered instead by the type IV pilus (T4P) (Chang et al. 2016). Not only for motility, the plant and human pathogenic bacteria also use T4P-mediated twitching motility to promote bacterial infections (Burrows 2012; Dungere et al. 2016; Corral et al. 2020). In the past decade, the transcription, post-transcription, and post-translation regulations of twitching motility have been well-documented from various bacterial systems (Burrows 2012; Craig et al. 2019). Among the twitching motility regulators, the PilZPA2960 protein is an original, stand-alone PilZ domain that exhibits both post-translation and protein-protein interaction capabilities in controlling T4P biogenesis and twitching motility (Alm et al. 1996; Guzzo et al. 2009). Afterwards, several PilZPA2960 homologs, i.e. PilZXac1133 and PilZ Xc1028, were identified from the flagellated, plant pathogenic Xanthomonas spp. (Guzzo et al. 2009). Interestingly, unlike PilZPA2960, the PilZXac1133 and PilZ Xc1028 proteins are potentially not involved in the establishment of twitching motility in their respective host systems (McCarthy et al. 2008; Guzzo et al. 2009). These earlier observations indicate the diversified roles of the homologous PilZPA2960 proteins in different bacterial species with flagella. In the previous study, we provided solid evidence to show that PilZLe3639 is indeed a homolog of PilZPA2960 and was able to regulate twitching motility in the non-flagellated soil bacterium of L. enzymogenes (Qian et al. 2009). In this aspect, PilZLe3639 seems to be involved in twitching motility prompting non-flagellated bacterium to move towards fungi to a close range for contact (Patel et al. 2010, 2011). The resulting
interaction of *L. enzymogenes*-fungi favours the non-flagellated bacteria to kill fungal pathogens to acquire nutrients, an aspect regarded as gaining adaptive advantage in natural niches. To our knowledge, this is the first report of the involvement of the widespread stand-alone PilZ domains in controlling twitching motility in non-flagellated bacteria, particularly in the biocontrol agent of phytopathogenic fungi.

In this study, we provide some further insights into how the stand-alone PilZ domains affect twitching motility in non-flagellated bacteria. In our case (Fig. 5c), we found that PilZ\textsubscript{Le3639} formed a binary complex with PilB\textsubscript{Le0708}, which is needed to transduce the signaling event for twitching motility. While the PilZ\textsubscript{Xac1133}-PilB\textsubscript{Xac3239} binding has been proved under the in vitro condition in the flagellated *Xanthomonas* spp. (Guzzo et al. 2009), whether such a protein-protein interaction contributes to twitching motility regulation is, however, not experimentally documented. Moreover, besides PilB\textsubscript{Xac3239}, PilZ\textsubscript{Xac1133} also directly interacts with FimX\textsubscript{Xac2398} which is a known regulator of T4P (Guzzo et al. 2009). Again, direct experimental evidence supporting the involvement of the PilZ\textsubscript{Xac1133}-FimX\textsubscript{Xac2398} complex in twitching motility is also lacking. Despite the hypothesis that PilZ\textsubscript{PA2960} most likely forms a complex with a T4P regulator and/or structural-component to co-activate the production of twitching motility in *P. aeruginosa*, both the PilZ\textsubscript{PA2960}-PilB\textsubscript{PA4526} and PilZ\textsubscript{PA2960}-FimX\textsubscript{PA4959} bindings, however, were not observed in this bacterium (Qi et al. 2012). Instead, FimX\textsubscript{PA4959} was shown to form a complex directly with PilB\textsubscript{PA4526} in *P. aeruginosa*, and this complex is vital for the “correct” polar-localization of both partners, which is essential for their co-involvement in the assembly of T4P (Jain et al. 2017). Therefore, the discovery of the PilZ\textsubscript{Le3639}-PilB\textsubscript{Le0708} binding presented in this study represents the first evidence that PilZ-PilB complex formation is indispensable for bacterial twitching motility. Besides, we observed that PilZ\textsubscript{Le3639} uses a crucial residue of W71 to bind with PilB\textsubscript{Le0708} and regulate bacterial twitching behavior, which provides a strong association between the PilZ\textsubscript{Le3639}-PilB\textsubscript{Le0708} binding and their co-regulation on twitching motility. What is the potent advantage for *L. enzymogenes* to form the PilZ\textsubscript{Le3639}-PilB\textsubscript{Le0708} complex? Our earlier works showed that *L. enzymogenes* indeed stimulated its own twitching motility when a nearby fungal pathogen is present but failed to generate twitching behavior under the nutrient-rich conditions (Zhou et al. 2015; Zhao et al. 2017). Based on these considerations, it is possible that upon sensing yet unidentified environmental or cellular stimuli, the non-flagellated *L. enzymogenes* becomes proficient in twitching motility. This attribute allows the biocontrol agent of crop fungal pathogens to access nutrients. It follows then that *L. enzymogenes* forms or disassembles the PilZ\textsubscript{Le3639}-PilB\textsubscript{Le0708} complex to move via twitching or to stop its motility when it lives in a nutrient-limited environment/presence of nearby fungi or nutrient-rich environment/absence of fungi, respectively. Such capacities could match the benefits and economic costs of *L. enzymogenes* in natural niches.

It is also noteworthy that the PilZ\textsubscript{Le3639} seems to interact with PilB\textsubscript{Le0708} but not PilT\textsubscript{Le3094}, a protein sharing 37% sequence similarity with PilB\textsubscript{Le0708} (Fig. 3b). At present, it is well recognized that PilB and PilT both function as ATPase, with the former supplying energy via ATP hydrolysis to promote T4P extension, while the latter inducing the retraction of T4P (Chiang et al. 2008). Therefore, the PilZ\textsubscript{Le3639}-PilB\textsubscript{Le0708} binding might support T4P extension, resulting in regulation of twitching motility. Besides, it was previously documented that for PilB\textsubscript{PA4526} of *P. aeruginosa*, both its ATPase activity and polar localization in cells (Jain et al. 2017) are essential for its function. Whether PilZ\textsubscript{Le3639} regulates twitching motility by binding with PilB\textsubscript{Le0708} to alter its ATPase activity and/or changing the PilB\textsubscript{Le0708} cellular localization remains unknown.

It is also noteworthy that recent studies showed that several transcription regulators or chemical signaling systems controlled the T4P-driven twitching motility in *L. enzymogenes* through a direct or indirect transcription regulation of genes or gene clusters responsible for pilus biogenesis (Chen et al. 2017, 2018; Feng et al. 2019). Unlike these cases, the present study uncovered a protein complex formed by PilZ\textsubscript{Le3639} and PilB\textsubscript{Le0708} that seems to affect pilus extension and hence play a key role in the formation of twitching motility in *L. enzymogenes*. These findings collectively suggest that the non-flagellated soil bacterium, *L. enzymogenes* has similarly designed multiple molecular strategies to modulate the twitching behavior in response to environmental stimuli.

Finally, according to earlier studies, T4P-mediated twitching motility enables *L. enzymogenes* to fully exhibit antagonistic capability against crop pathogens (Xia et al. 2018). The motile behavior helps *L. enzymogenes* to predate on fungi to derive nutrients and to colonize on the plant/soil surface and/or fungal mycelium. In this viewpoint, the fundamental insights presented in this study might be helpful to increase the biocontrol efficiency of *L. enzymogenes* in crop field by promoting twitching motility through engineering a more stable PilZ-PilB complex formation.

**Conclusions**

While the mechanisms by which flagellated bacteria control the formation of type IV pilus and/or twitching motility have been studied extensively, those in non-flagellated bacteria remain mostly unknown. In the present study, we showed the
mechanism by discovering the PilZ\textsubscript{Le3639}-PilB\textsubscript{Le0708} complex formation required for twitching motility in the non-flagellated, biocontrol bacterium, \textit{L. enzymogenes}. The binding between PilZ\textsubscript{Le3639} and the pilus-extension related motor protein PilB\textsubscript{Le0708} seems to be specific, as PilZ\textsubscript{Le3639} failed to interact with other pilus-retraction associated motor protein, PilT\textsubscript{Le3094} that shares 37% sequence similarity with PilB\textsubscript{Le0708}. These findings led us to propose that the PilZ\textsubscript{Le3639}-PilB\textsubscript{Le0708} binding seems to influence the process of T4P extension in a non-flagellated, soil bacterium. Our findings not only expand our current knowledge on the mechanistic actions about how bacteria without flagella control the flagella-independent twitching behavior, but also reveal that the biocontrol \textit{L. enzymogenes} likely engineers the PilZ-PilB complex to enhance twitching capacity. With such mechanism \textit{L. enzymogenes} is able to search and predate on nearby fungal pathogens, leading to crop protection.

**Methods**

**Bacterial strains, plasmids and growth conditions**

The bacterial strains and plasmids used in the present study are listed in the Additional file 1: Table S1. The \textit{Escherichia coli} strains that are used for plasmid construction were grown in Luria-Bertani (LB) medium with appropriate antibiotics at 37 °C. \textit{L. enzymogenes} strains were cultivated in LB medium or 1/10 Tryptic Soy Broth (TSB) at 28 °C. When required, appropriate antibiotics (kanamycin, Km, and gentamicin, Gm) were added into the media.

**Genetic methods**

A double-crossover homologous recombination approach was used to generate an in-frame deletion mutant of PilZ\textsubscript{Le3639} as described previously (Qian et al. 2012). The primers used in this assay are listed in the Additional file 1: Table S2. In brief, the corresponding primers were employed to amplify the flanking regions of the target gene and cloned into the suicide vector pEX18Gm (Additional file 1: Table S1). The final construct was transformed into the wild-type OH11 by electroporation and verified by PCR.

Chromosomal complementation or residue replacement was generated based on the double-crossover homologous recombination, as described previously (Xu et al. 2018). In brief, different primer pairs (Additional file 1: Table S2) were used to amplify DNA fragments containing the coding region and the flanking regions of each gene with or without point mutations by PCR. The purified PCR products were cloned into the suicide vector pEX18Gm to create a target construct (Additional file 1: Table S1), followed by transformation into the wild-type OH11 or mutants by electroporation. The selection of positive colonies and PCR confirmation were similar to those described above.

**Twitching motility assay**

We investigated twitching phenotype according to our earlier studies (Zhou et al. 2015; Xia et al. 2018). In brief, the wild-type OH11 and its derivatives were inoculated at the edge of a sterilized coverslip containing 1/20 tryptic soy agar (TSA) with 1.8% agar. Following a 24 h incubation at 28 °C, the margin of the test bacterial colonies on the microscope slide was observed under a light microscope. The twitching motility of \textit{L. enzymogenes} was designated as motile cells or cell clusters growing away from the original colony (Zhou et al. 2015).

**Protein expression and purification**

The coding region of PilZ\textsubscript{Le3639} and PilB\textsubscript{Le0708} was amplified by PCR with the primers listed in the Additional file 1: Table S2 and cloned into plasmid pET30a or pMAL-p2x to generate the PilZ\textsubscript{Le3639}-His\textsubscript{6} and PilB\textsubscript{Le0708}-MBP protein fusion, respectively (Additional file 1: Table S1). For protein expression and purification, each resulting vector was transformed into \textit{E. coli} BL21 (DE3) (Additional file 1: Table S1). The resulting strains were cultivated in LB medium (with 25 μg/mL Km) overnight at 37 °C. Two mL of the overnight culture was transferred into 200 mL of fresh LB at 37 °C and grown with shaking at 220 rpm, until reaching an OD\textsubscript{600} of 0.5. Isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma, USA) was added to a final concentration, 0.8 mM. The culture was incubated for an additional 4 h at 28 °C. The cells were collected by centrifugation (12,500 rpm) at 4 °C and resuspended in 20 mL of phosphate-buffered saline (PBS) lysis buffer with 10 mM protease inhibitor (PMSF, Sigma, USA). The resulting cells were lysed with 30-min sonication (Sonifier 250; Branson Digital Sonifier, Danbury, USA), and the crude cell extracts were centrifuged at 12,500 rpm at 4 °C for 25 min. Soluble proteins containing the PilZ\textsubscript{Le3639}-His\textsubscript{6} were collected and mixed with pre-equilibrated Ni\textsuperscript{2+} resin (GE Healthcare, Shanghai, China) for 1 h at 4 °C. The PilZ\textsubscript{Le3639}-His\textsubscript{6} were subsequently cloned into the broad-host vector pBBR1-MCS5 (Additional file 1: Table S1). The final constructed plasmid was transformed into the mutant by electroporation and verified by PCR.

Plasmid-based complementation assay was carried out as described previously (Qian et al. 2013). Briefly, different primer pairs listed in the Additional file 1: Table S2 were used to amplify the DNA fragment containing the coding region and the predicted promoter of the target gene by PCR, and the target DNA fragment was subsequently cloned into the broad-host vector pBBR1-MCS5 (Additional file 1: Table S1). The final constructed plasmid was transformed into the mutant by electroporation and verified by PCR.

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placed in a column and washed with the re-suspension buffer with 30 mM imidazole. The purified proteins were finally eluted in 250 mM imidazole. The supernatant (soluble proteins containing the PilB<sub>Le0708</sub>-MBP) was passed through 1 mL amylose resin (New England Biolabs) that retained the PilB<sub>Le0708</sub>-MBP protein. The column was washed with 200 mL of PBS buffer, and subsequently, 30 mL 10 mM maltose elution buffer was added into the column for protein elution. The protein concentration was determined using a BCA protein assay kit (Sangon Biotech, Shanghai, China), and the purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The GST-Clp fusion protein was purified according to our earlier study (Xu et al. 2018).

**Bacterial adenylate cyclase two-hybrid (BACTH) assay**

The BACTH system was used to test the direct interactions of two proteins of interest at the E. coli background (Ouellette et al. 2017). In brief, the coding region of PilZ<sub>Le</sub>3639 was cloned into pUT18c to generate the fusion protein comprising PilZ<sub>Le</sub>3639 and the T18 fragment. The coding genes of other test proteins were individually cloned to pKT25 to make each test protein fused with the T25 fragment. Each recombinant pKT25 plasmid along with the pUT18c-PilZ<sub>Le</sub>3639 plasmid were co-transformed into the E. coli BTH101 cells. The resulting E. coli strains were cultivated on LB agar plates with 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside (X-gal) at 50 μM. The physical interaction of T18 and T25-fused proteins was due to heterodimerization. The latter processes of these hybrid proteins resulted in functional complementation between T25 and T18 fragments, which in turn activate the synthesis of cyclic AMP (cAMP). cAMP binds with the transcription factor CRP to stimulate the transcription of several reporter genes, including genes of the lac operons whose product degrade X-gal resulting in a blue colony formation (Ouellette et al. 2017). The LacZ activity (β-galactosidase activity) was quantified as described previously (Ouellette et al. 2017).

**Microscale thermophoresis, MST**

The affinity of PilZ<sub>Le</sub>3639-His<sub>6</sub> or GST-Clp binding with c-di-GMP was determined by MST, a powerful technique for quantifying the protein-ligand and protein-protein interactions, using Monolith NT.115 (NanoTemper Technologies, Germany) as described previously (Xu et al. 2018; Han et al. 2020). In brief, the purified GST-Clp or PilZ<sub>Le</sub>3639-His<sub>6</sub> was labeled with the fluorescent dye NT-647-NHS (Nano Temper Technologies) via amine conjugation. Constant concentration (500 μM) of the labeled target protein in a standard MST buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 0.05% Tween 20) was titrated against increasing concentrations of c-di-GMP, which were dissolved in diethylpyrocarbonate-treated water. The MST premium-coated capillaries (Monolith NT.115 MO-K005, Germany) were used to load the samples into the MST instrument at 25 °C using 80% MST power, and 20% LED power. FNorm was plotted on a linear y-axis in per mil (‰) against the total concentration of the titrated partner on a log<sub>10</sub> x-axis, as reported earlier (Seidel et al. 2013). The experiment was performed in triplicate. Data were analyzed using Nanotemper Analysis software 2.2.4.4577 (NanoTemper Technologies).

**Supplementary information**

**Supplementary information** accompanies this paper at https://doi.org/10.1186/s42483-020-00054-x.

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**Additional file 1: Table S1.** Strains and plasmids used in this study. **Table S2.** Primers used in this study.

**Abbreviations**

BACTH: Bacterial adenylate cyclase two-hybrid system; c-di-GMP: Bis(3′,5′)-cyclic diguanosine monophosphate; HSAF: Heat-stable antifungal factor; MST: Microscale thermophoresis; T4P: Type IV pilus

**Acknowledgments**

Not applicable.

**Authors’ contributions**

GQ conceived the project. GQ and SH designed experiments. LL, MZ carried out experiments. LL, MZ, DS, SH, and GQ analyzed data and prepared figures and Tables. LL and GQ wrote the manuscript draft. GQ, AMF, and SHC revised the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**

Not applicable.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors have no conflicts of interest to declare.

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