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# 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 Bruijn 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).

*L. enzymogenes* is a plant-associated, soil proteobacterium that acts as a predator of crop fungal pathogens (Qian et al. 2009). To prey on nearby fungi, *L. enzymogenes* 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, HSAF (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 *L. enzymogenes* 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 Römmling et al. 2013, 2005). The first defined PilZ domain is PilZ<sub>PA2960</sub> that was found in *Pseudomonas aeruginosa*, a human pathogen and model flagellated bacterium (Alm et al. 1996). The PilZ<sub>PA2960</sub> protein has only one domain, PilZ (Alm et al. 1996; Guzzo et al. 2009), which controls T4P formation and twitching behavior. PilZ<sub>PA2960</sub> regulates the secretion of PilA polymers to the cell surface (Alm et al. 1996). At the mechanistic level, while PilZ<sub>PA2960</sub> appears to regulate the T4P formation via protein-protein interactions, the PilZ<sub>PA2960</sub>-binding partners are yet to be characterized (Galperin and Chou 2020). PilZ<sub>Xac1133</sub> from the flagellated, pathogenic *Xanthomonas citri* is homologous to the PilZ<sub>PA2960</sub> protein. Crystal structural analyses revealed that PilZ<sub>Xac1133</sub>, like PilZ<sub>PA2960</sub>, lacks two conserved signature motifs, RxxxR and [D/N] xSxxG, that are required for c-di-GMP binding (Guzzo et al. 2009; Li et al. 2009, 2011). PilZ<sub>Xac1133</sub> not only binds with PilB<sub>Xac3239</sub> but also with the degenerated EAL domain of FimX<sub>Xac398</sub>. FimX<sub>PA4959</sub> protein of *P. aeruginosa*, a homolog of FimX<sub>Xac398</sub>, has also been found to be an essential regulator of T4P formation and twitching motility (Huang et al. 2003; Guzzo et al. 2009). The discovery of these various protein-protein interactions implies that PilZ<sub>Xac1133</sub> is involved in regulating T4P formation; however, no corresponding phenotypic evidence is available. Moreover, PilZ<sub>Xac1133</sub>, unlike PilZ<sub>PA2960</sub>, did not seem to control twitching motility as described previously (Guzzo et al. 2009). In agreement, *X. campestris* PilZ<sub>Xc1028</sub>, which is almost identical to PilZ<sub>Xac1133</sub>, was found to play only a minor role in the twitching behavior (McCarthy et al. 2008). A similar situation has also been observed in the non-flagellated *Neisseria meningitidis*

(Carbonnelle et al. 2005). Therefore, PilZ<sub>PA2960</sub> and its homologs appear to play diversified roles in modulating the formation of T4P and/or twitching motility in different bacterial species with and without flagella.

A search of the genome of strain OH11 led us to discover Le3639, a homologous PilZ<sub>PA2960</sub> protein that is referred to as PilZ<sub>Le3639</sub> herein. PilZ<sub>Le3639</sub> shares a very high (76–83%) sequence similarity with PilZ<sub>PA2960</sub> and contains no conserved c-di-GMP-binding signature motifs, as described above (Fig. 1a). The objective of this work is to determine whether PilZ<sub>Le3639</sub> can affect the twitching motility of the non-flagellated *L. enzymogenes*, and if yes, what is the underlying mechanism? Herein, we showed that PilZ<sub>Le3639</sub> is indeed involved in controlling the twitching motility of *L. enzymogenes*. We further found that PilZ<sub>Le3639</sub> directly interacted with PilB<sub>Le0708</sub>, which is essential for twitching motility in *L. enzymogenes*. Moreover, PilZ<sub>Le3639</sub> seems to use the same residue critical for its role in twitching motility and interaction with PilB<sub>Le0708</sub>. 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.

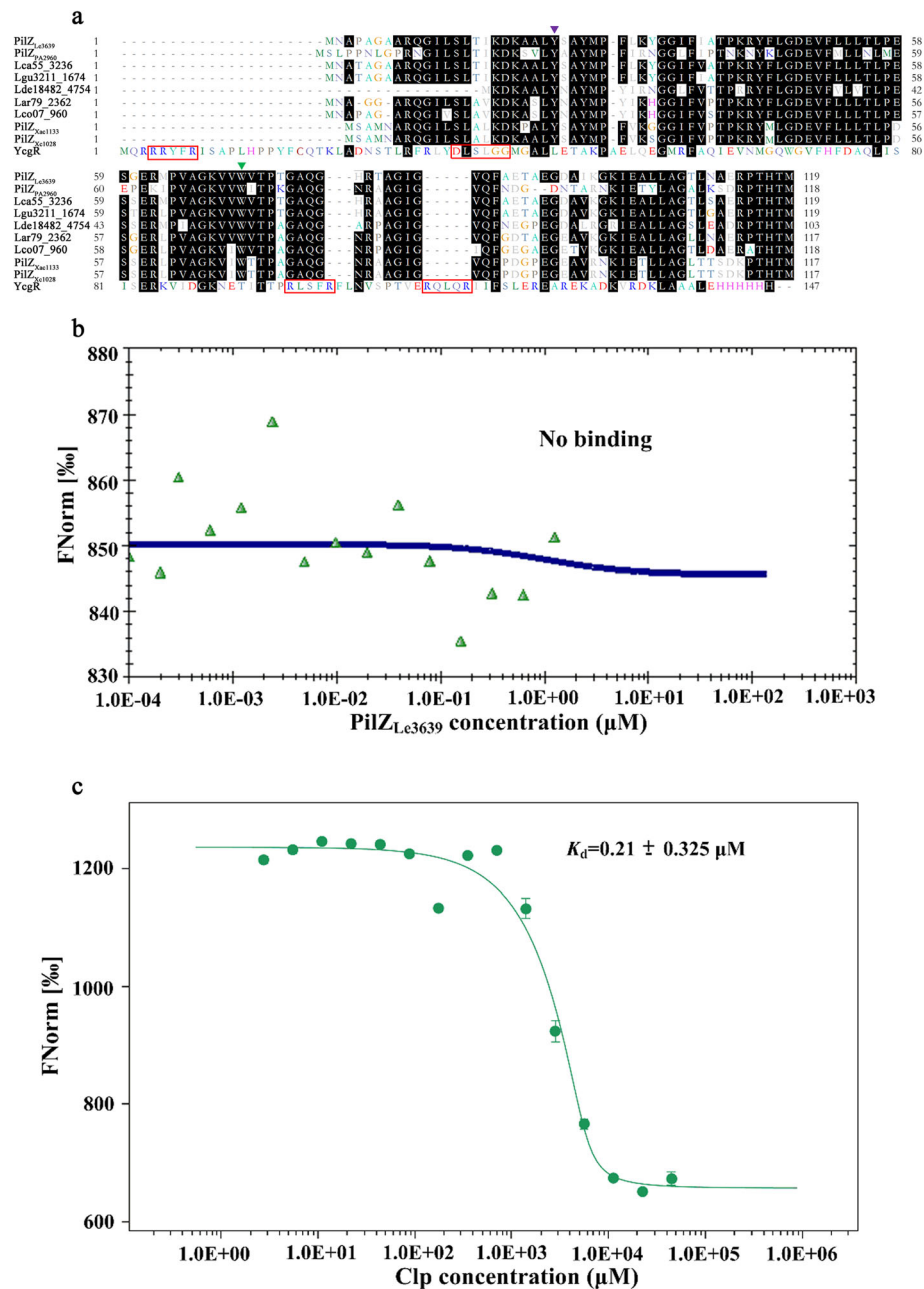
## Results

### PilZ<sub>Le3639</sub> is a stand-alone PilZ domain unable to bind c-di-GMP

A genomic search of strain OH11 by using PilZ<sub>PA2960</sub> as the bait enabled us to identify PilZ<sub>Le3639</sub>, a homologous protein in *L. enzymogenes* with a stand-alone PilZ domain (Fig. 1a) that shared 76%, 82%, and 83% sequence similarity with PilZ<sub>PA2960</sub>, PilZ<sub>Xac1133</sub>, and PilZ<sub>Xc1028</sub>, respectively. The homologous PilZ<sub>Le3639</sub> proteins were also present in several selected *Lysobacter* species (Fig. 1a). Multiple sequence alignment revealed that PilZ<sub>Le3639</sub> and its homologs from other selected *Lysobacter* species all lacked the conserved c-di-GMP-binding motifs described above (Fig. 1a). To experimentally validate this finding, we purified the PilZ<sub>Le3639</sub>-His fusion protein and subjected it to the microscale thermophoresis (MST) assay. We found no binding signals between the c-di-GMP and PilZ<sub>Le3639</sub>-His (Fig. 1b) could be observed, while c-di-GMP bound well to the control protein Clp (with a  $K_d$  of 0.21  $\mu$ M), a known c-di-GMP-binding transcription factor according to our earlier work (Fig. 1c; Xu et al. 2018). These results suggest that the non-flagellated *Lysobacter* species carry a conserved stand-alone PilZ domain that does not appear to interact with c-di-GMP as evidenced by PilZ<sub>Le3639</sub>.

### PilZ<sub>Le3639</sub> is essential for modulating the twitching motility of *Lysobacter*

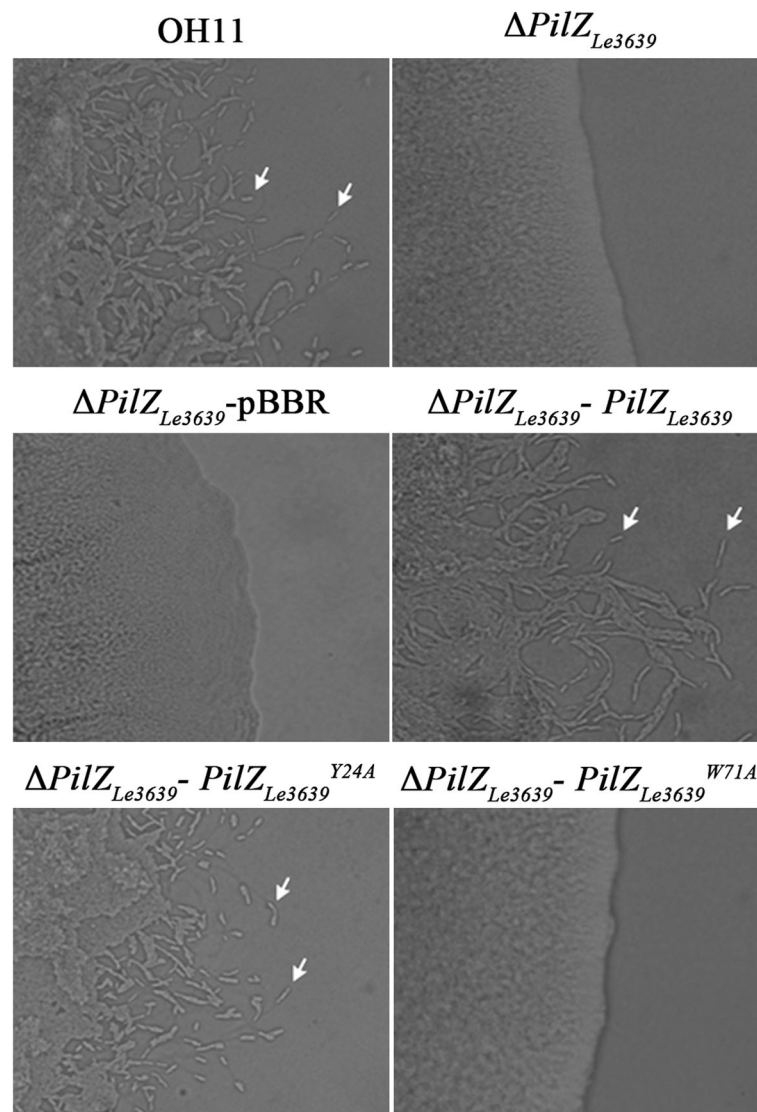
To investigate whether PilZ<sub>Le3639</sub> exhibits a role in affecting the *Lysobacter* twitching motility, we generated



**Fig. 1** PilZ<sub>Le3639</sub> is a stand-alone PilZ domain and unable to bind c-di-GMP. **a** Multiple sequence alignment of the PilZ<sub>Le3639</sub>-like proteins. PilZ<sub>Le3639</sub>, PilZ<sub>PA2960</sub>, Lca55\_3236, Lgu3211\_1674, Lde18482\_475, Lar79\_2362, Lco07\_960, PilZ<sub>Xac1133</sub> and PilZ<sub>Xc1028</sub> indicated these stand-alone PilZ domains from *Lysobacter enzymogenes* OH11, *Pseudomonas aeruginosa* PA01, *L. capsii* 55, *L. gulosus* 3.2.11, *L. defluvi* DSM18482, *L. arseniciresistens* ZS79, *L. concretions* Ko07, *Xanthomonas citri* and *X. campestris*, respectively. YcgR (PDB entry 5Y6G), is a c-di-GMP-binding protein from *E. coli*. The RxxxR and [D/N]xSxxG that are required for c-di-GMP binding (Guzzo et al. 2009) are highlighted by red boxes. Two conserved residues (Y24 and W71) in the selected stand-alone PilZ domains are indicated by purple and green triangles, respectively. **b** Microscale thermophoresis (MST) assay showed PilZ<sub>Le3639</sub> failed to bind with c-di-GMP, while **c** Clp, a known c-di-GMP-binding protein (Xu et al. 2018), showed moderate binding affinities with c-di-GMP determined by MST

$\Delta$ PilZ<sub>Le3639</sub>, 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<sub>Le3639</sub> completely abolished this twitching phenomenon (Fig. 2). The twitching motility defect in the PilZ<sub>Le3639</sub> mutant was



**Fig. 2** PilZ<sub>Le3639</sub> and its residue W71 both played essential roles in the formation of twitching motility by *L. enzymogenes*. Mutation of *pilZ*<sub>Le3639</sub> in wild-type OH11 led to a complete defect in forming twitching motility, as no twitching cells (indicated by white arrows) were observed at the margin of strain colony. Complementation of the wild-type *PilZ*<sub>Le3639</sub> or a variant carrying a Y24-A24 substitution (indicated as Y24A) into the mutant both rescued this motile deficiency. Introducing the empty vector (pBBR1-MCS5, abbreviated as pBBR) or the *pilZ*<sub>Le3639</sub> variant possessing a W71-A71 replacement (indicated as W71A) failed to rescue the twitching deficiency of the *pilZ*<sub>Le3639</sub> mutant

fully rescued by complementation with a plasmid-borne *PilZ*<sub>Le3639</sub> (Fig. 2) but not by an empty plasmid (Fig. 2). These results imply the crucial role of PilZ<sub>Le3639</sub> in triggering 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 executing the biological function of PilZ<sub>Xac1133</sub>. To figure out whether they also play a similar role in PilZ<sub>Le3639</sub>, we generated two individual mutants of Y24A (A is an abbreviation of Alanine) and W71A, respectively, and complemented these *PilZ*<sub>Le3639</sub> 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*<sub>Le3639</sub> 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<sub>Le3639</sub> is required for twitching motility, and the amino residue of W71 is essential for displaying such a function.

#### Binding of PilZ<sub>Le3639</sub> with PilB<sub>Le0708</sub> is required for twitching motility

To understand how PilZ<sub>Le3639</sub> modulates the *Lysobacter* twitching motility, we screened a total of 19 pilus-associated structural or regulatory proteins (Xia et al.



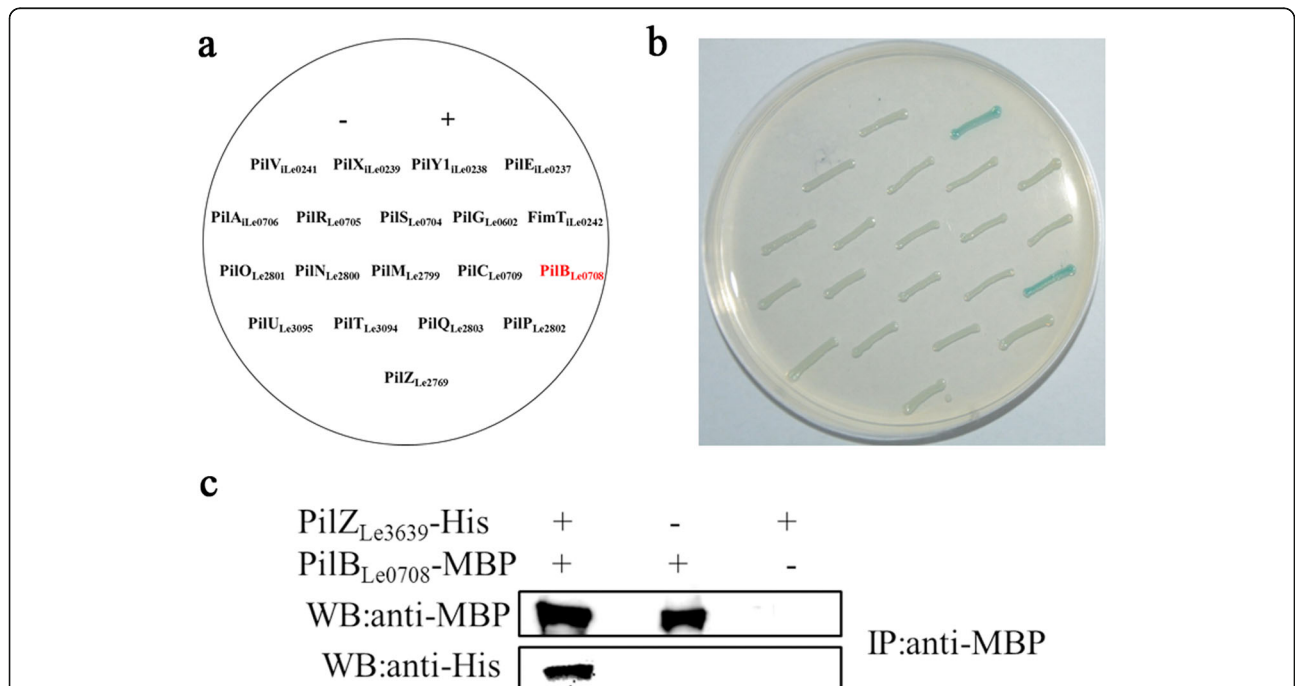
2018) to identify the direct PilZ<sub>Le3639</sub>-binding partner(s) (Fig. 3a). For screening, we employed the bacterial adenylate cyclase two-hybrid (BACTH) system (Ouellette et al. 2017). Each of the 19 genes was individually cloned into the pKT25 vector to generate the prey pKT25 constructs. Along with the bait plasmid pUT18c-PilZ<sub>Le3639</sub>, 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 PilZ<sub>Le3639</sub> 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 PilB<sub>Le0708</sub> interacted directly with PilZ<sub>Le3639</sub> (Fig. 3b). To validate this observation, we further tested the ability of PilZ<sub>Le3639</sub>-His to pull down GST-PilB<sub>Le0708</sub>, which was indeed the case since we observed a positive signal (Fig. 3c). These results reveal that PilZ<sub>Le3639</sub> directly interacted with PilB<sub>Le0708</sub> of *L. enzymogenes*.

Multiple sequence alignment showed that PilB<sub>Le0708</sub> 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<sub>Xac3239</sub>

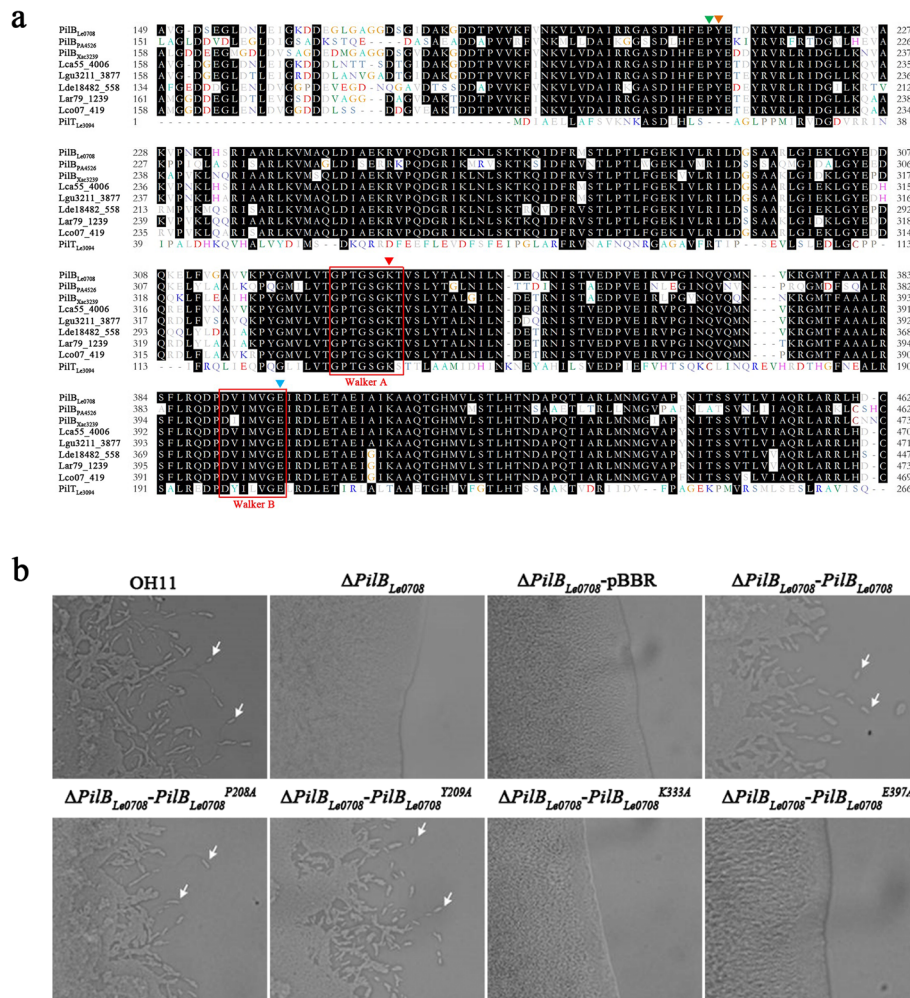
were previously shown to play potential key roles in forming the PilB<sub>Xac3239</sub>-PilZ<sub>Xac1133</sub> complex (Guzzo et al. 2009). Therefore, we constructed the PilB<sub>Le0708</sub> variants with a K333A or E397A substitution in the wild-type chromosome and subsequently tested the ability of 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 PilB<sub>Le0708</sub>, 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 PilB<sub>Le0708</sub>, 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 PilZ<sub>Le3639</sub>-PilB<sub>Le0708</sub> binding pair appears to correlate well with their functional outcomes in regulating the formation of twitching motility in *L. enzymogenes*.

**W71 of PilZ<sub>Le3639</sub> is required for PilZ<sub>Le3639</sub>-PilB<sub>Le0708</sub> interaction**

To provide molecular details for the PilZ<sub>Le3639</sub>-PilB<sub>Le0708</sub> binding, we tried to identify key residue(s) of PilZ<sub>Le3639</sub> or



**Fig. 3** PilZ<sub>Le3639</sub> directly interacted with PilB<sub>Le0708</sub>, a characterized pilus motor protein. **a, b** pKT25-PilZ<sub>Le3639</sub> 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 PilB<sub>Le0708</sub> with PilZ<sub>Le3639</sub> 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 PilZ<sub>Le3639</sub> with PilB<sub>Le0708</sub> in vitro

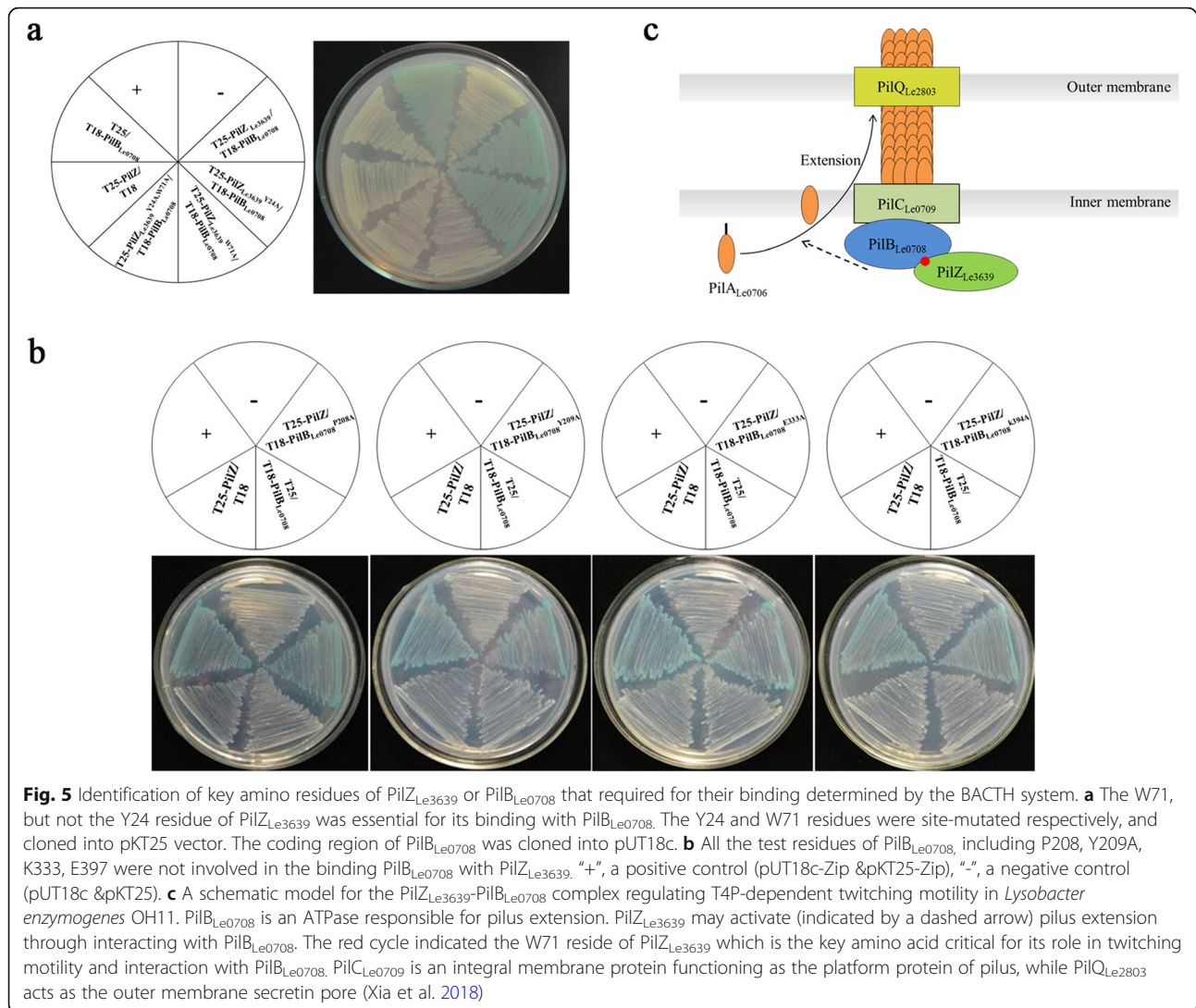


**Fig. 4** The K333 and E397 residues were required for the regulation of *PiIB*<sub>Le0708</sub> in the formation of twitching motility in *L. enzymogenes* OH11. **a** Multiple sequence alignment of the *PiIB*<sub>Le0708</sub>-like proteins. *PiIB* proteins acted as ATPase to supply energy via ATP hydrolysis to promote the extension of type IV pilus (T4P). *PiIB*<sub>Le0708</sub>, *PiIB*<sub>PA4526</sub>, *PiIB*<sub>Xac3239</sub>, *Lca55\_4006*, *Lgu3211\_3877*, *Lde18482\_558*, *Lar79\_1239*, *Lco07\_419*, indicated these *PiIB* proteins from *Lyso bacter enzymogenes* OH11, *Pseudomonas aeruginosa* PA01, *Xanthomonas citri*, *L. capsisi* 55, *L. gumosus* 3.2.11, *L. defluvi* DSM18482, *L. arseniciresistens* Z579, and *L. concretionis* Ko07, respectively. *PiIT*<sub>Le3094</sub> served as the *PiIT* protein from *L. enzymogenes* OH11, which functioned as ATPase to supply energy via ATP hydrolysis to elicit the retraction of T4P. “Walker A” and “Walker B” are two known motifs that are essential for the ATPase activity of the *PiIB* or *PiIT* family proteins. The conserved P208, Y209, K333 and E397 residues are indicated by green, orange, red and blue triangles, respectively. **b** Involvement of the K333 and E397 residues in the regulation of *PiIB*<sub>Le0708</sub> in twitching formation. Mutation of *PiIB*<sub>Le0708</sub> in wild-type OH11 fully impaired twitching motility, while complementation of the plasmid-borne *pilB*<sub>Le0708</sub> rescued this defect. At the wild-type background of strain OH11, chromosomal replacement of K333 to A333 (K333A) or E397 to A397 (E397A), but not P208-A208 (P208A) and Y209-A209 (Y209A) abolished the strain in producing twitching cells

*PiIB*<sub>Le0708</sub> that are important for their binding and function. For this purpose, we first selected the residues of W71 and Y24 of *PilZ*<sub>Le3639</sub> as a comparable pair, because we already showed that the former was necessary for the involvement of *PilZ*<sub>Le3639</sub> in twitching motility, while the latter was not (Fig. 2). Using the BACTH system, we found that the *PilZ*<sub>Le3639</sub> W71A variant exhibited almost entirely impaired binding capability, while the *PilZ*<sub>Le3639</sub> Y24A variant did not seem to demonstrate any binding with *PiIB*<sub>Le0708</sub> (Fig. 5a). These results collectively indicate that W71 is a key residue of *PilZ*<sub>Le3639</sub> in determining its

binding affinity with *PiIB*<sub>Le0708</sub> and to regulate the in vivo twitching motility.

To map the residues of *PiIB*<sub>Le0708</sub> that are essential for influencing its direct interaction with *PilZ*<sub>Le3639</sub>, we tested the binding of the *PilZ*<sub>Le3639</sub> with several *PiIB*<sub>Le0708</sub> variants (*PiIB*<sub>Le0708</sub><sup>K333A</sup>, *PiIB*<sub>Le0708</sub><sup>E337A</sup>, *PiIB*<sub>Le0708</sub><sup>P208A</sup>, and *PiIB*<sub>Le0708</sub><sup>Y209A</sup>) through the BACTH system and found that, like the native *PiIB*<sub>Le0708</sub>, all the tested *PiIB*<sub>Le0708</sub> variants seemed to be able to bind well with *PilZ*<sub>Le3639</sub> under the testing conditions (Fig. 5b). These results suggest that while K333 and E397 of



PilB<sub>Le0708</sub> are required for its own regulation in twitching motility, both of them are not likely involved in the PilB<sub>Le0708</sub>-PilZ<sub>Le3639</sub> 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; Dunger 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 PilZ<sub>PA2960</sub> 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 PilZ<sub>PA2960</sub> homologs, i.e. PilZ<sub>Xac1133</sub> and PilZ<sub>Xc1028</sub>, were identified from the flagellated, plant pathogenic *Xanthomonas* spp. (Guzzo et al. 2009). Interestingly, unlike PilZ<sub>PA2960</sub>, the PilZ<sub>Xac1133</sub> and PilZ<sub>Xc1028</sub> 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 PilZ<sub>PA2960</sub> proteins in different bacterial species with flagella. In the previous study, we provided solid evidence to show that PilZ<sub>Le3639</sub> is indeed a homolog of PilZ<sub>PA2960</sub>, and was able to regulate twitching motility in the non-flagellated soil bacterium of *L. enzymogenes* (Qian et al. 2009). In this aspect, PilZ<sub>Le3639</sub> 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<sub>Le3639</sub> formed a binary complex with PilB<sub>Le0708</sub>, which is needed to transduce the signaling event for twitching motility. While the PilZ<sub>Xac1133</sub>-PilB<sub>Xac3239</sub> 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<sub>Xac3239</sub>, PilZ<sub>Xac1133</sub> also directly interacts with FimX<sub>Xac2398</sub>, which is a known regulator of T4P (Guzzo et al. 2009). Again, direct experimental evidence supporting the involvement of the PilZ<sub>Xac1133</sub>-FimX<sub>Xac2398</sub> complex in twitching motility is also lacking. Despite the hypothesis that PilZ<sub>PA2960</sub> 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<sub>PA2960</sub>-PilB<sub>PA4526</sub> and PilZ<sub>PA2960</sub>-FimX<sub>PA4959</sub> bindings, however, were not observed in this bacterium (Qi et al. 2012). Instead, FimX<sub>PA4959</sub> was shown to form a complex directly with PilB<sub>PA4526</sub> 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<sub>Le3639</sub>-PilB<sub>Le0708</sub> 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<sub>Le3639</sub> uses a crucial residue of W71 to bind with PilB<sub>Le0708</sub> and regulate bacterial twitching behavior, which provides a strong association between the PilZ<sub>Le3639</sub>-PilB<sub>Le0708</sub> binding and their co-regulation on twitching motility. What is the potent advantage for *L. enzymogenes* to form the PilZ<sub>Le3639</sub>-PilB<sub>Le0708</sub> 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<sub>Le3639</sub>-PilB<sub>Le0708</sub> 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<sub>Le3639</sub> seems to interact with PilB<sub>Le0708</sub>, but not PilT<sub>Le3094</sub>, a protein sharing 37% sequence similarity with PilB<sub>Le0708</sub> (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<sub>Le3639</sub>-PilB<sub>Le0708</sub> binding might support T4P extension, resulting in regulation of twitching motility. Besides, it was previously documented that for PilB<sub>PA4526</sub> of *P. aeruginosa*, both its ATPase activity and polar localization in cells (Jain et al. 2017) are essential for its function. Whether PilZ<sub>Le3639</sub> regulates twitching motility by binding with PilB<sub>Le0708</sub> to alter its ATPase activity and/or changing the PilB<sub>Le0708</sub> 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<sub>Le3639</sub> and PilB<sub>Le0708</sub> 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<sub>Le3639</sub>-PilB<sub>Le0708</sub> complex formation required for twitching motility in the non-flagellated, biocontrol bacterium, *L. enzymogenes*. The binding between PilZ<sub>Le3639</sub> and the pilus-extension related motor protein PilB<sub>Le0708</sub> seems to be specific, as PilZ<sub>Le3639</sub> failed to interact with other pilus-retraction associated motor protein, PilT<sub>Le3094</sub> that shares 37% sequence similarity with PilB<sub>Le0708</sub>. These findings led us to propose that the PilZ<sub>Le3639</sub>-PilB<sub>Le0708</sub> 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 *L. enzymogenes* likely engineers the PilZ-PilB complex to enhance twitching capacity. With such mechanism *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 *Escherichia coli* strains that are used for plasmid construction were grown in Luria-Bertani (LB) medium with appropriate antibiotics at 37 °C. *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<sub>Le3639</sub>*, 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. After that, the *Lysobacter* transformants were selected on LB plates supplemented with Km and Gm. Positive colonies were grown in LB medium for 6 h without antibiotics and subsequently plated on LB agar carrying 10% (w/v) sucrose and Km to select the double-crossover colonies. The final in-frame deletions were verified by PCR using the primers listed in the Additional file 1: Table S2.

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.

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 *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<sub>Le3639</sub> and PilB<sub>Le0708</sub> 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<sub>Le3639</sub>-His<sub>6</sub> and PilB<sub>Le0708</sub>-MBP protein fusion, respectively (Additional file 1: Table S1). For protein expression and purification, each resulting vector was transformed into *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<sub>600</sub> 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 sonications (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<sub>Le3639</sub>-His<sub>6</sub> were collected and mixed with pre-equilibrated Ni<sup>2+</sup> resin (GE Healthcare, Shanghai, China) for 1 h at 4 °C. The PilZ<sub>Le3639</sub>-His<sub>6</sub> were

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>Le3639</sub> was cloned into pUT18c to generate the fusion protein comprising PilZ<sub>Le3639</sub> 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>Le3639</sub> 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 process 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>Le3639</sub>-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>Le3639</sub>-His<sub>6</sub> was labeled with the fluorescent dye NT-647-NHS (NanoTemper 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>.

**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

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#### 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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