Supplemental Material

The FilZ Protein Contains a Single PilZ Domain and Facilitates the Swarming Motility of *Pseudoalteromonas* sp. SM9913

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Table S1 Bacterial strains and plasmids used in this study.

Strain or plasmid	Description[a]	Source
Strains		
SM9913	Wild type	[26]
Δ*filZ*	*filZ* gene deletion mutant of SM9913	This study
Δ*2230*	*PSM_A2230* gene deletion mutant of SM9913	This study
Δ*filZ*Δ*2230*	Deletion mutant of the DNA region containing *filZ* and *PSM_A2230* genes	This study
Δ*0915*	*PSM_A0915* gene deletion mutant of SM9913	This study
9913(pEV)	Wild type strain harboring pEV plasmid	This study
Δ*filZ*(pEV)	Δ*filZ* mutant strain harboring pEV plasmid	This study
Δ*filZ*(pEV^{filZ})	Complemented strain of the Δ*filZ* mutant	This study
Δ*filZ*(pEV$^{filZ\text{-}R13A}$)	Δ*filZ* mutant strain expressing the mutant protein FilZ-R13A	This study
Δ*0915*(pEV^{0915})	Complemented strain of the Δ*0915* mutant	This study
Δ*0915*(pEV^{filZ})	Δ*0915* mutant strain expressing protein FilZ	This study
Δ*0915*(pEV$^{filZ\text{-}R13A}$)	Δ*0915* mutant strain expressing protein FilZ-R13A	This study
Δ*0915*(pEV)	Δ*0915* mutant strain harboring pEV plasmid	This study
E. coli MW3064	Donor strain for conjugation	[54]
E. coli DH5α	Strain for cloning	Vazyme
E. coli BL21(DE3)	Strain for high level expression of heterologous proteins	Vazyme
Plasmids		
pK18-*mobsacB*-Ery	Suicide vector for gene knockout, Kan^r, Ery^r	[54]
pK18-*filZ*	pK18*mobsacB*-Ery containing the homologous arms of *filZ* gene	This study
pK18-*2230*	pK18*mobsacB*-Ery containing the homologous arms of *PSM_A2230* gene	This study
pEV	Cloning vector for gene complementation, Amp^r, Cm^r	[38]
pEV^{filZ}	pEV plasmid expressing FilZ	This study
pEV$^{filZ\text{-}R13A}$	pEV plasmid expressing the mutant protein FilZ-R13A	This study
pEV^{0915}	pEV plasmid expressing protein A0915	This study
pKT25	The constructed plasmid in two-hybrid system	[35]
pUT18C	The constructed plasmid in two-hybrid system	[35]
pKT25-*2230*	The constructed plasmid in two-hybrid system, expressing the fusion protein T25-A2230	This study
pKT25-*cheA*	The constructed plasmid in two-hybrid system, expressing the fusion protein T25-CheA	This study
pKT25-*fliM$_p$*	The constructed plasmid in two-hybrid system, expressing the fusion protein T25-FliM$_p$	This study
pKT25-*fliM*	The constructed plasmid in two-hybrid system, expressing the fusion protein T25-FliM	This study
pUT18C-*2230*	The constructed plasmid in two-hybrid system, expressing the fusion protein T18-A2230	This study
pUT18C-*cheY*	The constructed plasmid in two-hybrid system, expressing the fusion protein T18-CheY	This study
pUT18C-*filZ*	The constructed plasmid in two-hybrid system, expressing the fusion protein T18-FilZ	This study
pUT18C-*filZ-R13A*	The constructed plasmid in two-hybrid system, expressing the fusion protein T18-FilZ-R13A	This study
pET22b-*filZ*	Expressing protein FilZ	This study
pET22b-*A2230*	Expressing protein A2230 with a C-terminal 6x His tag	This study
pGEX4T-1-*filZ*	Expressing protein FilZ with an N-terminal GST tag	This study

pGEX4T-1-*filZ-R13A*	Expressing protein FilZ-R13A with an N-terminal GST tag	This study

[a]Amp^r, ampicillin resistance; Ery^r, erythromycin resistance; Kan^r, kanamycin resistance; Cm^r, chloramphenicol resistance.

Table 2 Primers used in this study[a].

Primer (s)	Sequence (s) (5'–3')
For two-hybrid assay	
pKT25-*cheA*-F/R	GACTCTAGAGGATCCCCGGATGAGCTTTGA AGTCGATGA/CGAATTCTTAGTTACTTAGGT ACTTATCCAGCGTAATGCTTTA
pKT25-*fliM*$_p$-F/R	GACTCTAGAGGATCCCCGGGTGAGCGATTT ATTATCCCA/CGAATTCTTAGTTACTTAGGTA CTTACCAGTCTAAGTCTACAC
pKT25-*fliM*-F/R	GACTCTAGAGGATCCCCGGATGAGTAAAAC GTTAAGCGC/CGAATTCTTAGTTACTTAGGT ACTTAATTACTCGTTGTATTGT
pKT25-*2230*-F/R	GACTCTAGAGGATCCCCGGATGAGTGAAGA TAAATTACT/CGAATTCTTAGTTACTTAGGTA CTTATATATCGCTTAACTCAT
pUT18C-*filZ*-F/R	GACTCTAGAGGATCCCCGGATGAGTAACCA ATCAGAAAC/ACTTAGTTATATCGATGAATT CGACTATGCATTGGTTTTTTTTG
pUT18C-*2230*-F/R	GACTCTAGAGGATCCCCGGATGAGTGAAGA TAAATTACT/ACTTAGTTATATCGATGAATTC GATTATATATCGCTTAACTCAT
pUT18C-*cheY*-F/R	GACTCTAGAGGATCCCCGGTTGGATAAAAA CATTAAAAT/ACTTAGTTATATCGATGAATT CGATTAACCTAAGCGTTCGAACA
pUT18C-*filZ-R13A*-F/R	ATCTTTGAGGTTTAAATTCCATGCTTTGTAA CTTCGTTTAGTTTCTGATTGGTTAC/GTAACC AATCAGAAACTAAACGAAGTTACAAAGCAT GGAATTTAAACCTCAAAGAT
For gene mutation and complementation	
pK18-*filZ*-up-F/R	GTAAAACGACGGCCAGTGCCAAGCTTAATG ATGGAAAACAAAAATC/AATAGCCTTTTCAG CAGGCATTACTCATAATTCTCTTCAA
pK18-*filZ*-down-F/R	TTGAAGAGAATTATGAGTAATGCCTGCTGA AAAGGCTATT/GTCATAAGATTAGTCACTGG GGATCCGCTTTTAGTTGTTTTAATGT
pK18-*2230*-up-F/R	GTAAAACGACGGCCAGTGCCAAGCTTACTT TGATTATGCCGAGCTG/GACCATTACGAGGT AAGAGCGCTTCTCTACATAAAACTAT
pK18-*2230*-down-F/R	ATAGTTTTATGTAGAGAAGCGCTCTTACCTC GTAATGGTC/GTCATAAGATTAGTCACTGGG GATCCAACCTCTCAAGCGAAAAGCT
pK18-*0915*-up-F/R	GTAAAACGACGGCCAGTGCCAAGCTTGTGC ATTATTTTTGACGCAA/CATTTTATTTTTCCT TCAAAGAGTTCCGGTTTACTAATAA
pK18-*0915*-down-F/R	TTATTAGTAAACCGGAACTCTTTGAAGGAA AAATAAAATG/GTCATAAGATTAGTCACTGG GGATCCTCAAGCCCTGGCACACTGCC
filZ-screen-F/R	AAAAGTGATGACCATGGGCG/AATGCGAAC AAGGCGAAATTTC
2230-screen-F/R	ACCAGCTCTTGGTTTTGTTGTT/CATCTTTAA ATTATCAATATCTCATCACTTTAGGT
0915-screen-F/R	CGCTACCCCATTATTAAAAATTCAAAG/ACT GGCAATCATCGCCCC

pEV^{filZ}-F/R	TAAAAAACACACAACAGGAGATCTATGAGT AACCAATCAGAAAC/GGTGGTGGTGGTGCTC GAGCTATGCATTGGTTTTTTTTG
pEV$^{filZ\text{-}R13A}$-F/R	ATCTTTGAGGTTTAAATTCCATGCTTTGTAA CTTCGTTTAGTTTCTGATTGGTTAC/GTAACC AATCAGAAACTAAACGAAGTTACAAAGCAT GGAATTTAAACCTCAAAGAT
pEV^{0915}-F/R	TAAAAAACACACAACAGGAGATCTGTGTTT GTTGATGCTGATAC/GGTGGTGGTGGTGCTC GAGTTATGCAATTGCATCAATAT
For heterologous protein expression	
pET22b-*filZ*-F/R	TAAGAAGGAGATATACATATGATGAGTAAC CAATCAGAAAC/GTGGTGGTGGTGGTGCTCG AGCTATGCATTGGTTTTTTTTG
pGEX4T-1-*filZ*-F/R	GGATCTGGTTCCGCGTGGATCCATGAGTAA CCAATCAGAAAC/ACGATGCGGCCGCTCGA GCTATGCATTGGTTTTTTTTG
pGEX4T-1-*filZ-R13A*-F/R	ATCTTTGAGGTTTAAATTCCATGCTTTGTAA CTTCGTTTAGTTTCTGATTGGTTAC/GTAACC AATCAGAAACTAAACGAAGTTACAAAGCAT GGAATTTAAACCTCAAAGAT
pET22b-*A2230*-F/R	TAAGAAGGAGATATACATATGATGAGTGAA GATAAATTACT/GTGGTGGTGGTGGTGCTCG AGTTATATATCGCTTAACTCAT
For real-time qPCR	
rpoD-rt-F/R	CGCATATTATTGACTGGTTAGGTG/CAAGGG TTGAGGGTTCATAGC
filZ-rt-F/R	ATTACGAGCCAGAGTCAGTTCAG/CCACACC ACCCACGCTAATG
2230-rt-F/R	CCTTCGTATGTGATTGGTAT/CTTGCTCTTCT GCTTCAA

[a]The underlined nucleotides represent restriction sites.

Table S3 (separate file).

Table S3 is available as a separate Excel file. The legend is given below.

Table S3 Information of bacteria involved in the phylogenetic analysis of FilZ homologs and the alignment of the lateral flagellar cluster regions containing the *filZ* gene. NA, not available.

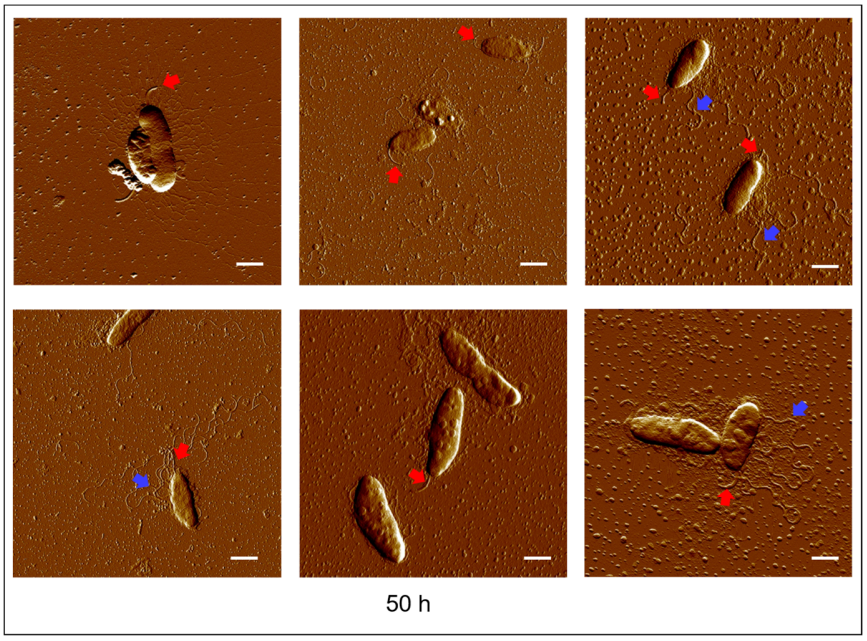

Figure S1. Observation of the production of flagella during strain SM9913 swarming at 50 h. Cells were extracted from the edge of the swarming colonies. The red arrow points to the polar flagellum, and the blue arrow points to the lateral flagella. Bar, 1 μm.

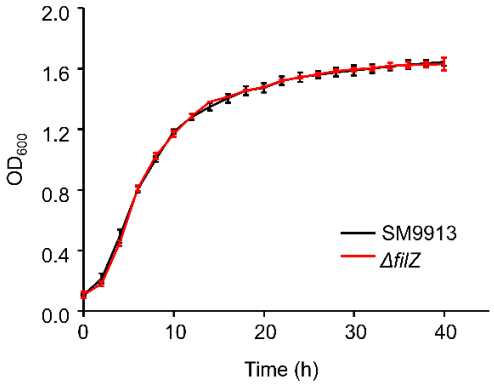

Figure S2. Growth curves of strain SM9913 and its mutant *ΔfilZ*. The strains were cultivated in marine LB broth at 15℃. The graph shows data from triplicate experiments (mean ± SD).

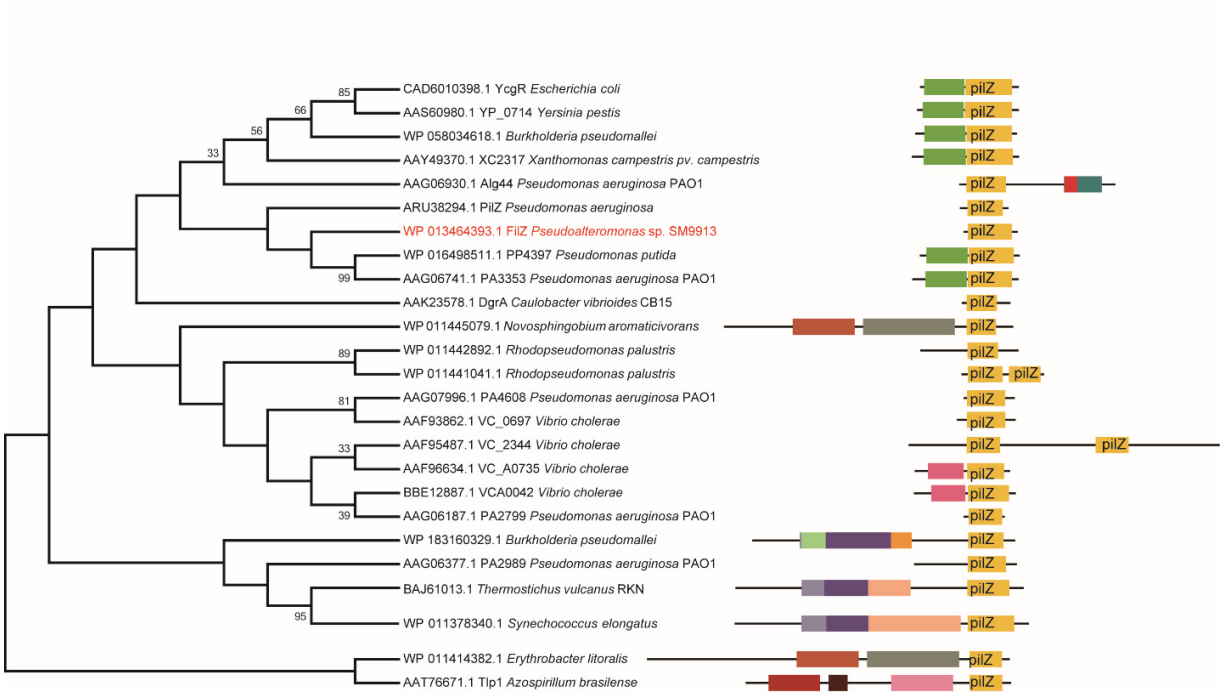

Figure S3. Phylogenetic analysis of bacterial PilZ domains. The left cladogram shows evolutionary relationships between PilZ domains inferred from maximum parsimony analysis of a multiple sequence alignment. The right diagrams schematize the domain organization of each PilZ protein inferred using RPS-BLAST analysis of the Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/cdd). FilZ of strain SM9913 was marked in red.

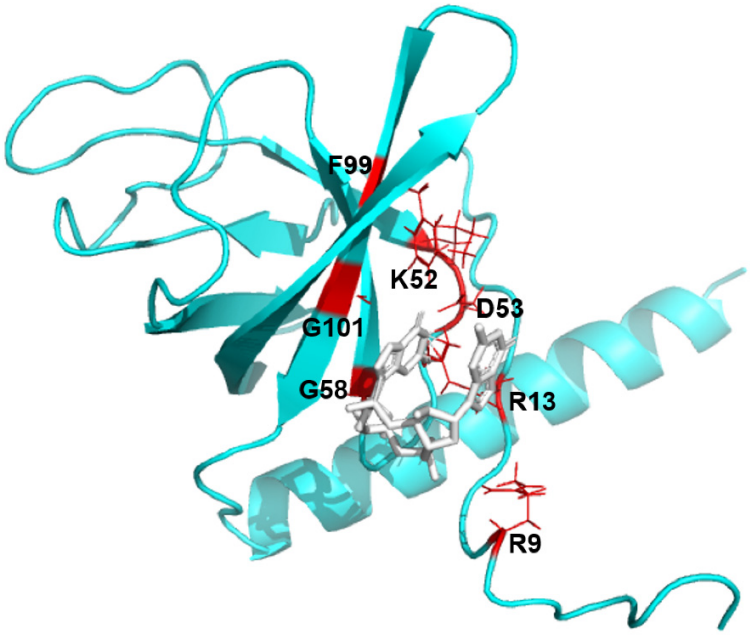

Figure S4. Predicted structure of protein FilZ (cyan) complexed with the c-di-GMP molecule (grey). The structure of FilZ was predicted by using I-Tasser (https://seq2fun.dcmb.med.umich.edu/I-TASSER-MR/) based on the structure of the C-terminal PilZ domain of protein YcgR (YcgR-PilZ) from *E. coli* (PDB ID 5Y6F). The possible residues of FilZ involved in binding c-di-GMP, including R9, R13, K52, D53, G58, F99 and G101, are marked in red.

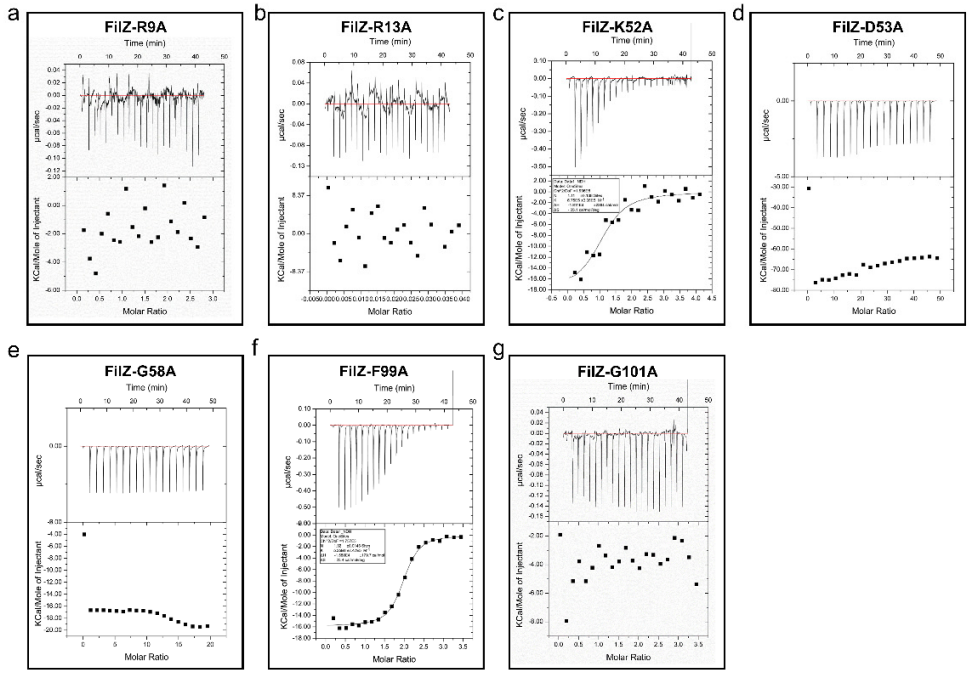

Figure S5. ITC detection of the c-di-GMP binding ability of recombinant FilZ mutants including FilZ-R9A (a), FilZ-R13A (b), FilZ-K52A (c), FilZ-D53A (d), FilZ-G58A (e), FilZ-F99A (f) and FilZ-G101A (g). The upper panels show the raw calorimetric data of the titration, and the lower panels show the corresponding integrated injection heats. The curves represent the best least-squares fits to the one binding site model. The graphs are representatives of at least three replicates.

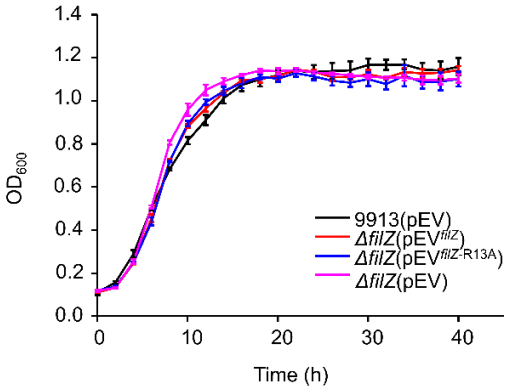

Figure S6. Growth curves of strains 9913(pEV), *ΔfilZ*(pEV^{filZ}), *ΔfilZ*(pEV$^{filZ\text{-}R13A}$) and *ΔfilZ*(pEV). All these strains were cultivated in marine LB broth at 15℃. The graph shows data from triplicate experiments (mean ± SD).

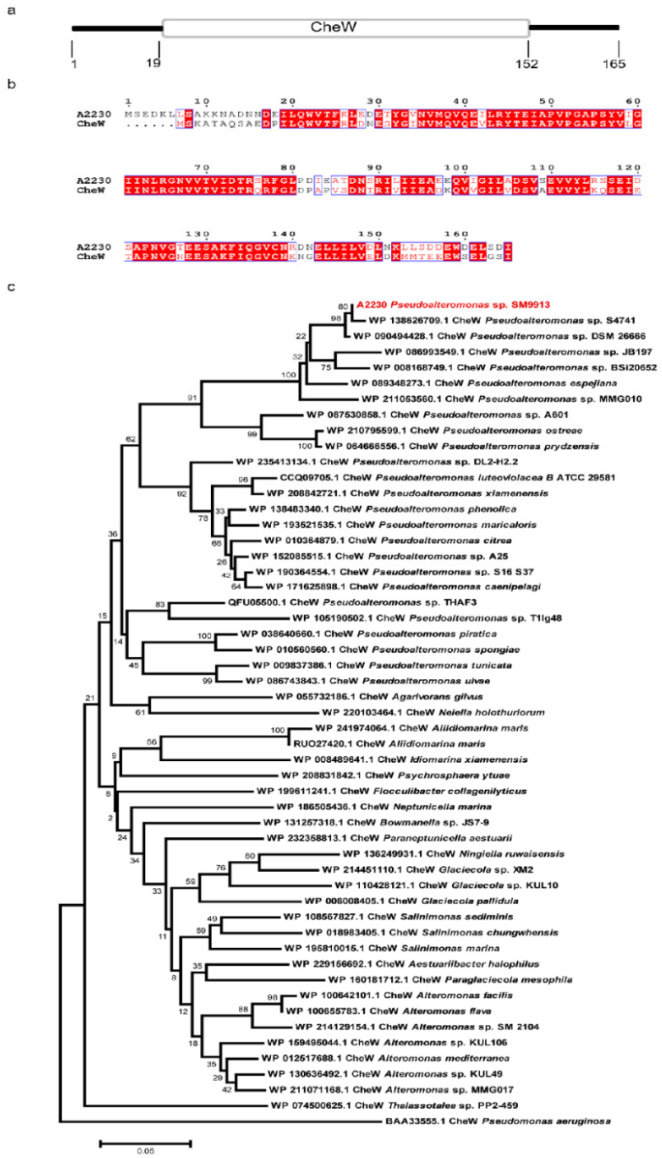

Figure S7. Sequence analysis of protein A2230. (a) Prediction of the protein domain in A2230 using the NCBI conserved domain database (https://www.ncbi.nlm.nih.gov/Structure/cdd). (b) Sequence alignment between protein A2230 and protein CheW (GenBank: BAA33555.1) of Pseudomonas aeruginosa. Sequence alignment shows a high identity (69%, 110/159 residues) between the two proteins. (c) Phylogenetic analysis of A2230 and its homologs obtained from the GenBank database by using MEGA 5. The phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates. The numbers at each tree node are the bootstrap values.

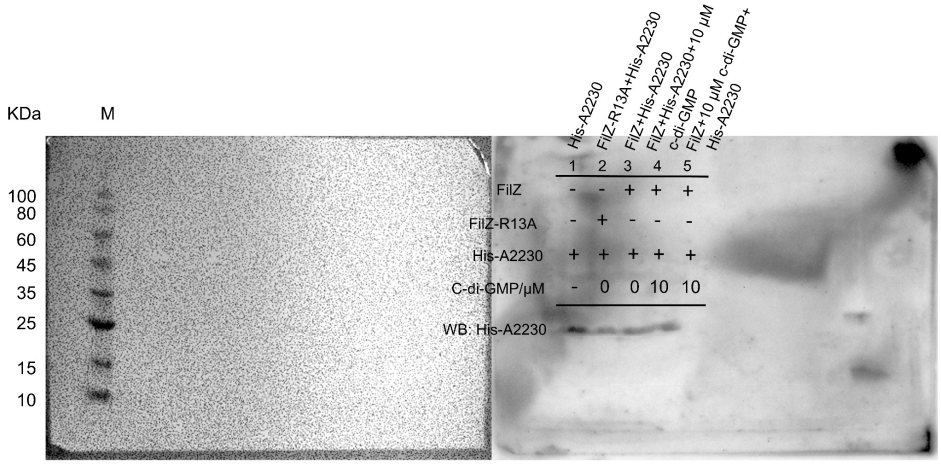

Figure S8. Pull down assay of the interaction between FilZ or FilZ-R13A and His-A2230 *in vitro* combined with Western Blot. The protein marker (the left) and the protein bands (the right) were from the same membrane of the Western Blot assay. Lanes 1 to 5, His-A2230 only, the mixture of FilZ-R13A and His-A2230, the mixture of FilZ and His-A2230, the addition of 10 μM c-di-GMP in the FilZ-A2230 mixture, and addition of A2230 in the FilZ-c-di-GMP mixture, respectively.

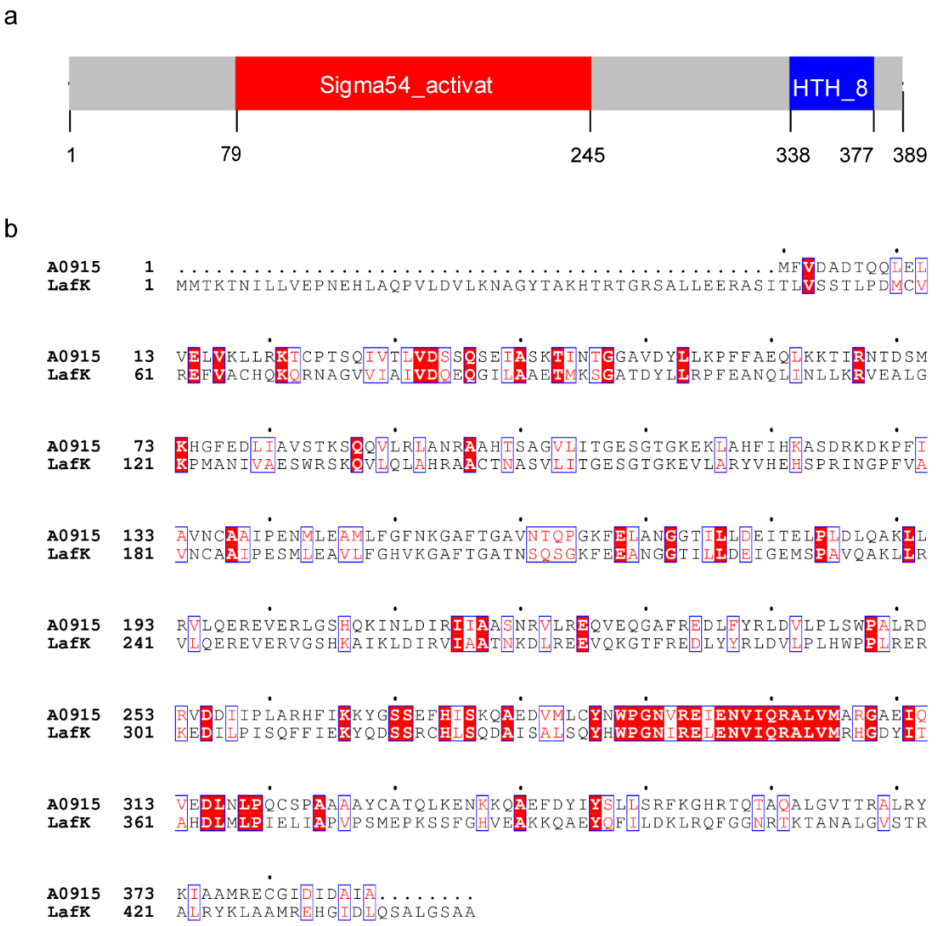

Figure S9. Sequence analysis of protein A0915. (a) Prediction of the protein domain in A0915 using the NCBI conserved domain database (https://www.ncbi.nlm.nih.gov/Structure/cdd). Protein A0915 contains an N-terminal Sigma54_activat domain (pfam00158, E-value 5.72e-102) and a C-terminal HTH_8 domain (pfam02954, 5.11e-04). (b) Sequence alignment between protein A0915 and protein LafK (GenBank: SUQ08311.1) of *Vibrio parahaemolyticus*. Sequence alignment shows a high identity (40.9%, 211/378 residues) between the two proteins.