1 **TagF-mediated repression of bacterial type VI secretion systems involves a direct**
2 **interaction with the cytoplasmic protein Fha**
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1 **Abstract**

2 The bacterial type VI secretion system (T6SS) delivers effectors into eukaryotic host
3 cells or toxins into bacterial competitor for survival and fitness. The T6SS is positively
4 regulated by the threonine phosphorylation pathway (TPP) and negatively by the
5 T6SS-accessory protein TagF. Here, we studied the mechanisms underlying TagF-mediated
6 T6SS repression in two distinct bacterial pathogens, *Agrobacterium tumefaciens* and
7 *Pseudomonas aeruginosa.* We found that in *A. tumefaciens,* T6SS toxin secretion and
8 T6SS-dependent antibacterial activity are suppressed by a two-domain chimeric protein
9 consisting of TagF and PppA, a putative phosphatase. Remarkably, this TagF domain is
10 sufficient to post-translationally repress the T6SS, and this inhibition is independent of TPP.
11 This repression requires interaction with a cytoplasmic protein, Fha, critical for activating
12 T6SS assembly. In *P. aeruginosa,* PppA and TagF are two distinct proteins that repress T6SS
13 in a TPP-dependent and -independent pathways, respectively. *P. aeruginosa* TagF interacts
14 with Fha1, suggesting that formation of this complex represents a conserved TagF-mediated
15 regulatory mechanism. Using TagF variants with substitutions of conserved amino acid
16 residues at predicted protein–protein interaction interfaces, we uncovered evidence that the
17 TagF–Fha interaction is critical for TagF-mediated T6SS repression in both bacteria. TagF
18 inhibits T6SS without affecting T6SS protein abundance in *A. tumefaciens*, but TagF
19 overexpression reduces the protein levels of all analyzed T6SS components in *P. aeruginosa*.
20 Our results indicate that TagF interacts with Fha, which in turn could impact different stages
21 of T6SS assembly in different bacteria, possibly reflecting an evolutionary divergence in
22 T6SS control.

23

1

2 **Introduction**

3 The type VI secretion system (T6SS) is a versatile weapon deployed by many bacterial
4 species to deliver diverse effector proteins into eukaryotic host cells or bacterial competitors.
5 The major target of T6SS antibacterial effectors include the membrane-, cell wall-, or nucleic
6 acid, some of these are shared by eukaryote-targeting effectors, *e.g.* membranes, while the
7 latter may have additional targets, *e.g.* actin cytoskeleton. The delivery and activity of these
8 T6SS toxins and effectors have a clear impact in interbacterial competition and/or
9 pathogenesis during eukaryotic host infection (1,2). The T6SS apparatus relies on
10 approximately 13-14 conserved core components to build a contractile phage tail-like
11 structure anchored to the bacterial cell envelope. To initiate the T6SS assembly, a TssJLM (or
12 TssLM) trans-membrane complex (3-6) serves as a docking site for the TssAEFGK baseplate
13 complex (7, 8), with TssK bridging the baseplate and the membrane complex. On the
14 baseplate, Hcp is polymerized in a tail tube-like structure and wrapped around by a TssB–
15 TssC outer sheath. In some studies it was proposed that TssA is responsible for initiating
16 sheath polymerization (9). Upon contraction of the sheath, the Hcp tube tipped by the
17 VgrG-PAAR puncturing device and the T6SS effectors associated with it (10,11) are
18 propelled across the cell envelope.

19 T6SS is regulated at multiple levels (12-15). A subset of T6SS gene clusters encode
20 orthologs of serine/threonine kinase PpkA, the cognate phosphatase PppA, and the
21 forkhead-associated (FHA) domain-containing proteins (16), which suggests the involvement
22 of a threonine phosphorylation (TPP) regulatory pathway in these bacteria (17-20). *P.*
23 *aeruginosa* H1-T6SS is post-translationally regulated, positively by PpkA and negatively by
24 the cognate phosphatase PppA. Such control occurs *via* threonine phosphorylation at the
25 Thr362 residue on a FHA domain-containing protein, Fha1, in *P. aeruginosa* (21) and

1 *Serratia marcescens* (20). Remarkably, the T6SS inner-membrane protein TssL and not Fha
2 was identified as the substrate of PpkA first in *Agrobacterium tumefaciens* (22) and recently
3 in *Vibiro alginolyticus* (23). In *A. tumefaciens,* TssL forms a stable complex with TssM
4 (4,6,24), which exhibits ATPase activity (5). Phosphorylated TssL recruits Fha to the TssM–
5 TssL complex for T6SS activation (22). In *P. aeruginosa*, four type VI secretion associated
6 genes, namely *tagQRST*, participate in post-translational regulation and act upstream of PpkA
7 to promote kinase activity and subsequent T6SS-dependent secretion (25,26). Remarkably,
8 the *P. aeruginosa* H1-T6SS can also be activated independently of Fha1 phosphorylation and
9 TPP activity by inactivating a negative regulator, TagF (19). Yet, the molecular basis
10 underlying the regulatory mechanisms of TagF-mediated T6SS repression has not been
11 addressed.

12 *A. tumefaciens* harbors one T6SS that is activated at both transcriptional and
13 post-translational levels when sensing acidity (22,27,28). Three T6SS effectors, including one
14 peptidoglycan amidase (Tae) and two DNases (Tde1 and Tde2), conferring antibacterial
15 activity, have been identified in this bacterium (29). Autointoxication is prevented through the
16 production of cognate immunity proteins. In *A. tumefaciens*, TagF and PppA are encoded as a
17 fusion protein named TagF-PppA, the overexpression of which abolishes Hcp secretion (22).
18 In this study, we investigated the TagF-PppA suppression mechanism and how this affects
19 T6SS-dependent secretion and antibacterial activity in *A. tumefaciens*. Our data indicate that
20 the TagF domain alone is sufficient to post-translationally repress the T6SS independently of
21 TPP. By performing protein–protein interaction studies, we identified that the cytoplasmic
22 T6SS core component Fha is the binding target for TagF. Using structural modeling, we
23 identified conserved TagF amino acid residues and demonstrated their importance in Fha
24 interaction and TagF-mediated repression of T6SS activity. Remarkably, these conserved
25 residues are also required for *P. aeruginosa* TagF in repressing the H1-T6SS activity and

interaction with Fha1. TagF may have evolved while keeping the Fha protein as a target for controlling and preventing T6SS assembly.

Results

Both TagF and PppA domains can repress type VI secretion and antibacterial activity at post-translational levels in *A. tumefaciens*

TPP regulation of T6SS has been demonstrated in only a few bacteria, including *A. tumefaciens* strain C58 (22). PpkA (Atu4330), TagF-PppA (Atu4331), and Fha (Atu4335), the three major components of the TPP pathway, are all encoded within the *imp* operon (Figure 1A) (22,27,30). Atu4331 is a fusion protein, which contains N- and C-terminal domains homologous to TagF (DUF2094) (11-219 aa) and PppA (244-470 aa), respectively (thus named TagF-PppA) (Figure 1B) (22). We previously reported that TagF-PppA plays a negative role in regulating Hcp secretion when TagF-PppA is overexpressed in *A. tumefaciens* (22). However, the molecular mechanism underlying this regulation is unknown. Thus, we first investigated the impact and respective role of individual domain from the TagF-PppA chimera on the TagF-PppA-mediated repression activity on T6SS activity. In *A. tumefaciens* strain C58, we separately produced the TagF domain located at the N terminus (1-230 aa) and the C-terminal region (231-471 aa) encompassing the PppA domain (Figure 1B). Overexpression of TagF-PppA, TagF, and tagged TagF-Strep in C58 completely abolished type VI secretion (Hcp, Tae and Tde1) (Figure 2A). The T6SS antibacterial activity is also shut down, as shown by counting surviving *E. coli* target cells. The number of survivors is indeed similar as when the attacker is an *A. tumefaciens* T6SS mutant, Δ*tssL* (Figures 2B). We also performed *in planta* interbacterial competition assay with an *A. tumefaciens* prey strain lacking the three T6SS toxins (Tae, Tde1 and Tde2) and cognate immunity proteins, Δ*3Tis* (29). Previous observation showed reduced number of viable prey cells when co-infected with

WT C58 (31). Similarly, here the survival of Δ*3TIs* was lower after co-infection with WT C58 harboring the vector pTrc200 (V). This was not seen when co-infecting with a T6SS inactive mutant Δ*tssL*-carrying pTrc200 (V), or C58 overexpressing the T6SS repressor TagF, *i.e.* TagF-PppA, TagF, or TagF-Strep (Figure 2C). Interestingly, overexpression of PppA alone reduced but did not abolish Hcp secretion (Figure 2A). The antibacterial activity of the PppA-overexpression strain was also not completely abolished, while modest antibacterial activity was detected with our *in planta* interbacterial competition assay (Figure 2C). In addition, the protein levels of all analyzed T6SS components encoded within the *imp* (including TssM, TssL, TssK, Fha, $TssC_{41}$, TssB, and TssA) and *hcp* (including ClpV and VgrG1) operons remained the same in all backgrounds tested, including overexpression of TagF-PppA, TagF, TagF-Strep, or PppA (Figure 2A). Taken together, these data show that in *A. tumefaciens* both TagF and PppA domains contribute the repressor function of TagF-PppA on T6SS effector secretion and antibacterial activity *via* a post-translational regulatory control.

Both TagF and PppA domains repress T6SS activity independently of PpkA-mediated TssL phosphorylation

To explore the possible mechanisms of TagF-PppA–mediated T6SS repression at the post-translational level, we analyzed the impact on TssL phosphorylation. TagF-PppA, TagF, TagF-Strep, and PppA were overexpressed in the Δ*tssL* mutant also expressing His-tagged TssL (TssL-His). This TssL variant is functional and mediates Hcp, Tae and Tde1 secretion (Figures S1A and S1B) (22). The TssL-His protein was purified by using Ni-NTA resins, and various *A. tumefaciens* strains were analyzed. The Phos-tag SDS-PAGE approach was used, which can detect two TssL-His protein bands, the lower band representing the

1 unphosphorylated TssL-His and the upper band representing the phosphorylated TssL-His
2 (*p*-TssL-His) (Figure 3A) (22). In all cases, the TssL-His protein displayed a similar
3 phosphorylation pattern (Figure 3A), which suggests that neither the full-length nor any
4 domain of TagF-PppA represses T6SS activity by controlling TssL phosphorylation.

5 To determine whether the phosphorylation level detected from an overexpressed TssL-His
6 is representative of an endogenous situation, we generated an antibody that specifically
7 recognizes phosphorylated TssL (*p*TssL) (Figure 3B). Phosphorylated TssL protein was
8 detected as a single protein band and found with the same abundance when comparing WT
9 C58 and strains overexpressing TagF-PppA, TagF, TagF-Strep, and PppA. However, the
10 *p*TssL protein band was not detected in Δ*tssL* and Δ*ppkA* mutant strains (Figure 3C). We
11 concluded that TagF-PppA represses T6SS activity independently of TssL phosphorylation in
12 *A. tumefaciens*.

13 It is intriguing that overexpression of PppA domain had no impact on TssL
14 phosphorylation, which is mediated by PpkA, but still could repress T6SS activity. To
15 determine that the TPP-independent repression of both TagF and PppA domains is not caused
16 by secondary effect linked to overexpression, we also examine the T6SS secretion and
17 antibacterial activity in a strain lacking the entire TPP, *i.e.* both *ppkA* and *tagF-pppA*. We
18 generated a Δ*ppkA*Δ*tagF-pppA* strain and separately overexpressed the TagF or PppA domain
19 in this mutant background. Interestingly, unlike Δ*ppkA* resulting in decreased type VI
20 secretion (22), the Δ*ppkA*Δ*tagF-pppA* mutant retained comparable type VI secretion activity
21 to that of the WT C58 (Figure 4A). Overexpression of each of TagF or PppA domain in
22 Δ*ppkA*Δ*tagF-pppA* abolished or greatly reduced the type VI secretion (Figure 4B), suggesting
23 that both TagF and PppA domains can inhibit type VI secretion in the absence of PpkA. As
24 expected, the antibacterial activity was also abolished when TagF or PppA domain was
25 overexpressed in Δ*ppkA*Δ*tagF-pppA* as shown by counting *E. coli* survivors and comparison
26 to what is observed when the attacker is a T6SS mutant, Δ*tssL* (Figure 4C). Interestingly,

1 while the Hcp and effector (Tde1 and Tae) secretion levels in Δ*ppkA*Δ*tagF-pppA* was
2 comparable to that of WT C58 (Figure 4A), only partial antibacterial activity was observed
3 (Figure 4C). This phenotype is consistent with a previous observation in *P. aeruginosa* and *S.*
4 *marcescens,* with Δ*pppA* showing reduced antibacterial activity despite elevated type VI
5 secretion (20,32,33). These data indicate that both the TagF and PppA domains play a role in
6 repressing the *A. tumefaciens* T6SS activity. This control is exerted at a post-translational
7 level and is independent of PpkA and TssL phosphorylation.

8

9 **TagF interacts with the forkhead-associated protein Fha of *A. tumefaciens* and *P.***
10 ***aeruginosa***

11 Next, we investigated how TagF-PppA represses T6SS activity *via* a post-translational
12 and TPP-independent pathway. We hypothesized that TagF-PppA may interact with the T6SS
13 core component(s) to prevent T6SS activation. One plausible candidate is the
14 forkhead-associated protein Fha because of its role in recruitment to a membrane-associated
15 complex both independently and dependently of TPP (19,21,22). Because TagF is the
16 common repressor known to suppress T6SS independently of TPP in both *A. tumefaciens* and
17 *P. aeruginosa,* we set up experiments to determine whether *A. tumefaciens* TagF-PppA and *P.*
18 *aeruginosa* TagF can interact with their cognate Fha. Yeast two-hybrid (YTH) experiments
19 revealed that both *A. tumefaciens* full-length TagF-PppA and the isolated TagF domain
20 specifically interact with Fha (Figure 5A). We also detected an interaction between the *P.*
21 *aeruginosa* H1-T6SS-encoded TagF and Fha1 by using both YTH (Figure 5B) and bacterial
22 two-hybrid (BTH) assays (Figure 5C). The data demonstrate that TagF directly interacts with
23 Fha likely interfering with its function and thereby preventing type VI secretion in both *A.*
24 *tumefaciens* and *P. aeruginosa.*

1

2 **Conserved amino acid residues in TagF are critical for TagF–Fha interaction**

3 Although Fha is the target for TagF in both *A. tumefaciens* and *P. aeruginosa,* the two

4 TagF proteins share only limited amino acid similarity (Figure 6A). Yet, 14 amino acids

5 residues are highly conserved among various TagF orthologs (Figures 6A and S3A). We

6 hypothesized that these residues may play important roles in TagF function. The structure of

7 *P. aeruginosa* TagF is presented as a homodimer (34). The self-interaction of *P. aeruginosa*

8 TagF was also confirmed by BTH and YTH (Figures 5C and S2A). In contrast, the *A.*

9 *tumefaciens* TagF does likely function as a monomer, as supported by several lines of

10 evidence. First, the TagF protein is mostly a monomer when analyzed by gel filtration *in vitro*

11 (Figure 6B). Second, *A. tumefaciens* TagF lacks the residues required for dimer formation in

12 *P. aeruginosa* [Val105 (V105), Leu169 (L169), Leu172 (L172), Ala173 (A173), and Leu195

13 (L195) in $TagF^{Pa}$] (Figure S3A) (34). Finally, no self-interaction could be found for *A.*

14 *tumefaciens* TagF using YTH (Figure S2B). Other conserved residues are not involved in *P.*

15 *aeruginosa* TagF homodimerization and were thus chosen for site-directed mutagenesis and

16 characterization of the potential interface with other interacting proteins, namely Fha. A total

17 of eight conserved residues [Gly8 (G8), Lys9 (K9), Asp15 (D15), Phe16 (F16), Ser79 (S79),

18 Asp81 (D81), Arg85 (R85), and Pro (P88) of *P. aeruginosa* TagF] form a specific loop, and

19 the side chain of Asp30 (D30) is outwardly exposed (Figure 6C). Therefore, these are

20 potential sites to interact with other proteins. In contrast, Phe60 (F60), Gly74 (G74), and

21 Leu139 (L139) are located inside the structure or the side chain wrapped in an internal

22 structure to prevent interaction with other proteins. We generated four mutants with amino

23 acid substitutions of alanine at GK (G8K9 in *P. aeruginosa* TagF; G22K23 in *A. tumefaciens*

24 TagF), DF (D15F16 in *P. aeruginosa* TagF; D29F30 in *A. tumefaciens* TagF), DW (D30W32

25 in *P. aeruginosa* TagF; D44W46 in *A. tumefaciens* TagF), and SDR (S79D81R85 in *P.*

1 *aeruginosa* TagF; S93D95R99 in *A. tumefaciens* TagF) (Figures 6A and 6C). We performed
2 YTH analysis with the various *A. tumefaciens* TagF variants to determine the roles of the
3 substituted residues in the TagF–Fha interaction. The interaction was completely lost in
4 $TagF^{GK}$, $TagF^{DW}$, and $TagF^{SDR}$ mutants (Figure 6D). In contrast, $TagF^{DF}$ and $TagF^{FD}$ with
5 mutations in two randomly selected non-conserved residues [Phe141Asp142 (F141D142) in *A.*
6 *tumefaciens* TagF] still retained full capacity to interact with Fha as compared with the WT
7 TagF (Figure 6D). Western blot analysis revealed that the protein abundance remained the
8 same or was even slightly higher for all analyzed TagF variants as compared to the WT TagF
9 (Figure S3B), which indicates that conserved $G^{22}K^{23}$, $D^{44}W^{46}$, and $S^{93}D^{95}R^{99}$ residues but not
10 $D^{29}F^{30}$ of *A. tumefaciens* TagF are critical for the TagF–Fha interaction.

11

12 **Loss of TagF–Fha interaction upon site-directed mutagenesis abolishes the repression of**
13 **T6SS activity in *A. tumefaciens***

14 To determine whether the TagF–Fha interaction is required for suppressing T6SS
15 activity, we engineered the previously described mutations in the TagF-Strep variants and
16 analyzed the impact of their overexpression on type VI secretion. As expected,
17 overexpression of the WT controls, TagF-Strep or $TagF^{FD}$-Strep, abolished Hcp, Tae and
18 Tde1 secretion. Remarkably, the secretion capacity remained high with overexpression of
19 $TagF^{GK}$-Strep, $TagF^{DF}$-Strep, $TagF^{DW}$-Strep and $TagF^{SDR}$-Strep in C58 (Figure 7A). Upon
20 western blot analysis, the protein levels of all TagF variants were comparable to that of WT
21 TagF-Strep and overexpression did not affect the protein abundance of other T6SS
22 components (Figure 7A). These data suggest that the conserved residues GK, DW, and SDR
23 of TagF are critical for TagF-mediated repression of type VI secretion via the TagF–Fha
24 interaction. Intriguingly, $TagF^{DF}$ retained full binding capacity with Fha but lost the ability to

repress type VI secretion, which suggests that $D^{29}F^{30}$ is not involved in binding Fha but is required for repressing T6SS activity. As expected, the type VI secretion activity of these TagF overexpression variants was consistent with their antibacterial activity (Figures 2B and 2C). The survival of *E. coli* was reduced to a level similar to that with the strain harboring the empty vector control when $TagF^{GK}$-Strep was overexpressed in Δ*ppkA*Δ*tagF-pppA* (Figures 4B and 4C), which confirms that $TagF^{GK}$-Strep lost the ability to repress T6SS-dependent antibacterial activity via a PpkA-independent pathway. To ensure the overexpression phenotypes of the mutant alleles also reflected when expressed at endogenous levels, we further generated chromosomal *tagF-pppA* alleles encoding the TagF amino acid substitution variants and further determined their T6SS suppression activity. All TagF amino acid substitution variants expressed from chromosomal alleles exhibited enhanced antibacterial activity (Figure 7B), which is consistent with the de-repressing T6SS activity demonstrated upon overexpression of these TagF variants (Figures 2B and 2C). However, our secretion assay could not detect significant difference on secretion levels of Hcp and two effectors (Tae and Tde1) of all analyzed *tagF-pppA* mutants as compared to wild-type C58 (Figure S4). Taken together, our data suggest that TagF negatively regulates T6SS *via* direct interaction with Fha in *A. tumefaciens*.

Overexpression of TagF causes reduced T6SS protein accumulation and abolishes T6SS antibacterial activity in *P. aeruginosa*

We then assessed whether these specific conserved amino acid residues of TagF required for binding to Fha and T6SS repression in *A. tumefaciens* are also required in *P. aeruginosa* TagF for $Fha1^{Pa}$ interaction and T6SS activity. We generated two alanine substitution mutants in $TagF^{Pa}$, namely $TagF^{Pa\text{-}GK}$ (G8 and K9) and $TagF^{Pa\text{-}SDR}$ (S79, D81 and

R85). Consistent with the results obtained with *A. tumefaciens*, TagF$^{Pa\text{-}GK}$ and TagF$^{Pa\text{-}SDR}$ mutants lost the interaction with Fha1^{Pa} even though their expression level was comparable to that of WT TagF^{Pa} in yeast (Figures 8A and S3C). To determine whether the TagF^{Pa}–Fha1^{Pa} interaction is critical for TagF-dependent H1-T6SS repression, WT TagF^{Pa}, Strep-tagged WT TagF^{Pa} and the TagF$^{Pa\text{-}GK}$ and TagF$^{Pa\text{-}SDR}$ variants were expressed from pRL662 in the *P. aeruginosa* Δ*retS* mutant, a constitutively H1-T6SS active strain (35). As expected, Hcp1 and Tse3 were secreted into the culture medium of Δ*retS* harboring the vector pRL662 (V), but H1-T6SS secretion was greatly reduced when TagF^{Pa} or TagF^{Pa}-Strep was overexpressed (Figure 8B). Furthermore, overexpression of TagF$^{Pa\text{-}GK}$-Strep or TagF$^{Pa\text{-}SDR}$-Strep did not repress Hcp1 and Tse3 secretion. Interestingly, in contrast to *A. tumefaciens* where overexpressed TagF mutant variants come in level comparable to the overexpressed WT form, in *P. aeruginosa* we observed significantly higher levels of WT TagF^{Pa}-Strep as compared with TagF$^{Pa\text{-}GK}$-Strep or TagF$^{Pa\text{-}SDR}$-Strep (Figure 8B). Furthermore, protein levels of all analyzed T6SS components (including secreted proteins Hcp1 and Tse3 and structural proteins TssB1 and TagJ1) were significantly reduced upon overexpression of WT TagF (TagF^{Pa} or TagF^{Pa}-Strep). Accordingly, protein levels of these T6SS components were restored to near-WT levels in the presence of TagF$^{Pa\text{-}GK}$-Strep and TagF$^{Pa\text{-}SDR}$-Strep in *P. aeruginosa* (Figure 8B).

Next, we performed antibacterial activity and showed that when TagF^{Pa}-Strep was overexpressed in PAKΔ*retS*, *E. coli* survival is similar to that for the T6SS-defective PAKΔ*retS*Δ*tssB1* mutant. In contrast, the expression of TagF$^{Pa\text{-}GK}$-Strep and TagF$^{Pa\text{-}SDR}$-Strep conferred similar antibacterial activity as that of PAKΔ*retS* (Figure 8C). Because the reduced protein level/stability of both TagF$^{Pa\text{-}GK}$-Strep and TagF$^{Pa\text{-}SDR}$-Strep is associated with loss of ability to interact with Fha1^{Pa}, we tested whether the presence or absence of Fha1^{Pa} affected TagF abundance. Strikingly, TagF^{Pa}-Strep protein level was significantly reduced in PAKΔ*retS*Δ*H1*, which lacks endogenous Fha1^{Pa}, as compared with PAKΔ*retS* (Figure 8D).

TagF^{Pa}-Strep protein levels could be partially restored upon co-expression of TagF^{Pa}-Strep and Fha1^{Pa}-HA in PAKΔ*retS*Δ*H1* (Figure 8D), which suggests that Fha1^{Pa} may play a role in stabilizing TagF^{Pa} in *P. aeruginosa*. Taken together, our results indicate that the TagF domain represses T6SS activity via interaction with Fha but with distinct mechanisms when comparing *A. tumefaciens* and *P. aeruginosa.*

Discussion

In the present study, we characterized the *A. tumefaciens* TagF-PppA and *P. aeruginosa* TagF and provide compelling evidence that TagF specifically interacts with Fha to repress type VI secretion and antibacterial activity independently of TPP. The loss of interaction with Fha is associated with loss of repression of T6SS activity in both cases. Yet, we observed differences in the nature and impact of TagF and its variants in repressing T6SS. This may have strategic consequences on how bacteria from various species communicate and respond to each other during T6SS-dependent bacterial warfare.

One remarkable difference is that TagF–Fha interaction has a different impact on T6SS protein abundance between *A. tumefaciens* and *P. aeruginosa*. A similar protein abundance of all TagF^{At} or TagF^{At}-Strep overexpressed (WT and all mutants) was observed in *A. tumefaciens*. In contrast, in *P. aeruginosa*, the protein level was significantly lower for TagF$^{Pa\text{-}GK}$-Strep or TagF$^{Pa\text{-}SDR}$-Strep than WT TagF^{Pa}-Strep (Figures 2A, 7A, and 8B). Furthermore, all analyzed T6SS proteins accumulated to a similar level in the presence or absence of endogenous or overexpressed TagF^{At} in *A. tumefaciens* (Figures 2A and 7A). However, the protein levels of *P. aeruginosa* H1-T6SS components (including secreted proteins Hcp1 and Tse3 and structural proteins TssB1 and TagJ1) were reduced with TagF^{Pa} or TagF^{Pa}-Strep overexpression but restored to near-WT levels in the presence of mutant TagF$^{Pa\text{-}GK}$-Strep or TagF$^{Pa\text{-}SDR}$-Strep (Figure 8B). Previous studies in *P. aeruginosa* suggested

1 that TagF^{Pa}-mediated T6SS repression is mediated *via* a post-translational regulation
2 mechanism (19). The evidence is based on the lack of influence on the expression of
3 *lacZ*-translational fusion to *fha1* or *tssA1* and on the levels of two secreted proteins, Hcp and
4 Tse1, in a Δ*tagF* mutant, which activates Hcp and Tse1 secretion. Instead of analyzing a *tagF*
5 mutant here, we used strains overexpressing TagF, and our data support that the *P.*
6 *aeruginosa* TagF-mediated post-translational repression occurs *via* influencing the protein
7 stability of T6SS components when TagF^{Pa} is expressed in excess amounts. Taken together,
8 Fha is a common target for TagF in repressing T6SS *via* post-translational regulation in both
9 *A. tumefaciens* and *P. aeruginosa*, but each have different strategies to exert this repression
10 activity.
11 The role of Fha in activating T6SS in both TPP-dependent and -independent pathways
12 may provide some clues to understand the mode of action by which TagF represses T6SS
13 activity. Fha1 but not its phosphorylated form is required for ClpV1^{Pa} foci formation and
14 TagF^{Pa}-mediated de-repression of type VI secretion in *P. aeruginosa* (19,21). Also, Fha
15 protein is a core component for T6SS in several bacteria lacking PpkA, PppA, and TagF
16 (21,22,30,36). Therefore, in addition to functioning as a scaffold protein specifically binding a
17 phosphothreonine protein, Fha may also serve as a core T6SS component likely *via*
18 interaction with one or multiple T6SS components to activate T6SS assembly and secretion.
19 TssM could be such a candidate because Fha1 foci formation is lost in a *P. aeruginosa*
20 Δ*icmF(tssM)* mutant (37). In *A. tumefaciens*, Fha specifically interacts with TssL at
21 phosphothreonine 14 (*p*T14) to associate with the TssM–TssL inner membrane complex and
22 recruit Hcp to interact with TssL for activating type VI secretion (4,22). Together with our
23 current knowledge of the T6SS assembly pathway (2,10,38), these studies indicate that Fha
24 plays a key role in T6SS assembly at step(s) before recruitment of Hcp and TssB–TssC for
25 tail polymerization. In *A. tumefaciens*, only WT TssL but not a TssL variant with the T14A
26 amino acid substitution could interact with Fha, as assessed by pulldown assay (22). Thus,

1 Fha may interact with other core component(s) of the T6SS in addition to TssL or Fha itself,
2 especially when PpkA is absent or not active. If so, TagF may compete with other T6SS core
3 components for interacting with Fha and thereby prevent Fha from binding to a T6SS
4 membrane-associated complex for stability (in *P. aeruginosa*) or activation of T6SS assembly
5 (in *A. tumefaciens*).

6 Of note, the conserved $G^{22}K^{23}$, $D^{44}W^{46}$, and $S^{93}D^{95}R^{99}$ residues but not $D^{29}F^{30}$ of *A.*
7 *tumefaciens* TagF are critical for the TagF–Fha interaction but all are required for repressing
8 T6SS activity. This result led us to propose that $D^{29}F^{30}$ may repress T6SS function
9 independently of binding to Fha. TagF may target Fha in repressing T6SS activity in two
10 steps. The first step is to bind Fha *via* an interface involving $G^{22}K^{23}$, $D^{44}W^{46}$, and $S^{93}D^{95}R^{99}$.
11 Upon binding, TagF interferes with Fha recruitment to the membrane-associated complex for
12 T6SS activation via $D^{29}F^{30}$ residues. Alternatively, it is possible that TagF can also target
13 other T6SS components in addition to Fha. Future work to elucidate the molecular details
14 underlying how TagF-Fha interaction influences T6SS activity or identifying additional
15 TagF-interacting partners may provide answers to distinguish between two possible
16 mechanisms.

17 Combining previous (19,21,22) and current findings, we propose distinct models for
18 TagF-mediated T6SS repression in *A. tumefaciens* and *P. aeruginosa* (Figure 9). In WT *A.*
19 *tumefaciens* when PpkA is active, the level of endogenous TagF-PppA is very low and the
20 protein does not bind to Fha, with no or little repression activity observed because Fha would
21 then bind to *p*TssL for triggering T6SS assembly and secretion (T6SS ON shown in the upper
22 left panel of Figure 9). On sensing an unknown signal, which may cause high accumulation of
23 TagF-PppA or suppression of the TPP pathway, TagF-PppA can interact with Fha *via* its
24 TagF domain to prevent it from binding to the membrane-associated complex and thus
25 preventing T6SS activation (T6SS OFF shown in the upper right panel of Figure 9). Because

1 TssM and TssL can form an inner-membrane complex in absence of TssL phosphorylation
2 (4,22), the TssM–TssL complex and the associated baseplate complex likely remain properly
3 assembled in the membrane when T6SS is suppressed by TagF-PppA. However, Hcp and
4 TssBC may not be polymerized into the tail-like structure, and effector proteins are not loaded
5 on the VgrG-PAAR spike for secretion. Because type VI secretion can be restored to the WT
6 level in the Δ*ppkA*Δ*tagF-pppA* mutant (Figure 4A), Fha likely also functions as a core T6SS
7 component *via* interaction with one or multiple T6SS components to activate T6SS assembly
8 and secretion in the absence of PpkA and TagF-PppA. This proposed mechanism also
9 explains the previous observation that type VI secretion is highly attenuated but not
10 completely abolished in the absence of PpkA (Δ*ppkA*) (22,30), because endogenous
11 TagF-PppA, albeit at a low level, can bind Fha, and only a fraction of the Fha pool is
12 available for recruitment to the T6SS core complex.

13 In *P. aeruginosa*, type VI secretion is significantly enhanced in Δ*pppA* or Δ*tagF* as
14 compared with the parental strain, and PppA phosphatase negatively regulates type VI
15 secretion in a TPP-dependent manner, whereas TagF represses type VI secretion
16 independently of TPP (19,21). Thus, in WT *P. aeruginosa* strain harboring both TPP
17 components and TagF, type VI secretion remains at low levels likely because of a series of
18 phosphorylation and dephosphorylation events as well as TagF interaction with a fraction of
19 the Fha1 pool (T6SS ON shown in the lower left panel of Figure 9). When TagF is expressed
20 in excess amounts, TagF interacts with Fha to prevent it from binding to T6SS components
21 and thus from activating T6SS assembly and subsequent secretion. Failure of recruiting Fha1
22 to the membrane-associated T6SS complex may send out a signal to trigger degradation of
23 cytoplasmic T6SS components and effectors (T6SS OFF shown in the lower right panel of
24 Figure 9). Our current study may also provide an explanation for the enhanced T6SS secretion
25 and antibacterial activity in a Δ*tagF* mutant in the presence or absence of PpkA (19,21).

1 Because Fha1 protein itself but not its phosphorylation is required for ClpV1^{Pa} foci formation
2 (19,21), non-phosphorylated Fha1 may remain active in binding membrane-associated T6SS
3 component(s), thus resulting in T6SS assembly and secretion in *P. aeruginosa*. With no TagF
4 functionally available (*i.e.,* in the absence of the protein or presence of a TagF mutant losing
5 Fha1 binding activity), all Fha1 is available for activating T6SS assembly and secretion.

6 In conclusion, our proposed molecular model may provide answers to the long-standing
7 question of how TagF mediates T6SS repression. We present compelling evidence suggesting
8 that TagF specifically interacts with Fha and that such binding prevents Fha from recruitment
9 to a T6SS membrane-associated complex. The TagF–Fha interaction has different impact in
10 different bacteria but ultimately prevents successful T6SS assembly. This study adds to our
11 understanding of how bacteria deploy TPP-dependent activation and TPP-independent
12 TagF-mediated repression mechanisms to control T6SS.

13

14 **Experimental procedures**

15 **Bacterial strains, plasmids, and growth conditions**

16 Strains, plasmids, and primer sequences used in this study are described in Tables S1 and S2.
17 The growth conditions were described in detail in Supporting Information S1.

18 **Plasmid construction and generation of in-frame deletion mutants**

19 All in-frame deletion mutants were generated in *A. tumefaciens* C58 via double crossover
20 using the suicide plasmid pJQ200KS (39) as previously described (4,27). The detailed
21 procedures for the construction of plasmids and mutant strains are described in Supporting
22 Information S1.

23 **Antibody production**

1 The specific antibody for phosphorylated TssL (*p*TssL) was generated against the 15-mer
2 peptide (7-SSWQDLP*p*TVVEITEE-21) containing the phosphorylated Thr 14 residue (22).
3 The *p*TssL epitope located at the N-terminal region of TssL was used for polyclonal antibody
4 production in rabbits.

5 ***A. tumefaciens* type VI secretion and antibacterial competition assays**

6 Type VI secretion assay was performed as described previously (4,27,29,30). To study type
7 VI secretion from *A. tumefaciens* grown in liquid medium, *A. tumefaciens* cells were grown in
8 liquid 523 medium for 16 hr at 25 °C. Cells were harvested and OD_{600} was adjusted to 0.1, *A.*
9 *tumefaciens* cells continued to grow in liquid AB-MES medium (pH 5.5) (40) at 25 °C for 6
10 hr. The cell suspension was centrifuged at 10,000 x g for 15 min at 4 °C; the resulting
11 supernatant was concentrated by trichloroacetic acid (TCA) precipitation and Hcp, Tae, and
12 Tde1 secretion monitored as previously described (4,27). *A. tumefaciens* antibacterial activity
13 assay using *E. coli* as target cells was performed as previously described (29). In brief,
14 overnight grown *A. tumefaciens* and *E. coli* strains harboring appropriate plasmids were
15 adjusted to OD_{600} 0.1 and incubated at 25°C for 4-5 hr prior to co-incubation. *A. tumefaciens*
16 and *E. coli* cells were harvested and OD_{600} was adjusted, mixed at 1:30 ratio (OD_{600} 0.01:0.3)
17 and spotted onto LB agar plates. Where applicable, the mixture was spotted onto a LB agar
18 plate containing 0.5 mM IPTG to induce expression from the pTrc200 plasmid. After 16 hr
19 post-incubation at 25 ^{o}C, the spots were harvested, serially diluted and plated on LB agar plate
20 containing appropriate antibiotic to quantify surviving *E. coli* cells by counting
21 colony-forming units (CFUs). *In planta* bacterial competition assay was performed as
22 previously described (29). Briefly, *A. tumefaciens* strains were transformed with gentamycin
23 (Gm), resistance conferred by the pRL662 plasmid or spectinomycin (Sp), resistance
24 conferred by the pTrc200 plasmid, for selecting surviving cells. The attacker (OD_{600} 5) and
25 target (OD_{600} 0.5) strains were mixed in 1/2 Murashige and Skoog (MS) medium (pH 5.7) at a

1 10:1 ratio and infiltrated into leaves of 6- to 7-week-old *N. benthamiana* plants by use of a
2 needleless syringe. After 24 hr incubation at room temperature, the infiltrated spot was
3 punched out, ground in 0.9% NaCl, serially diluted, and plated in triplicates on LB agar
4 containing appropriate antibiotic to select for the target cells. All assays were performed with
5 at least two independent experiments and each with two biological replicates; or three
6 independent experiments and each with one or two biological replicates.

7 ***P. aeruginosa* type VI secretion and antibacterial competition assays**

8 *P. aeruginosa* type VI secretion assay was performed as previously described (41). In brief, *P.*
9 *aeruginosa* strains harboring appropriate plasmids were grown in tryptone soy broth (TSB)
10 overnight at 37 °C under agitation. Cells were harvested and sub-cultured to an OD_{600} of 0.1,
11 then growth was continued in TSB to early stationary phase at 37 °C for 4-5 hr to OD_{600} 5.
12 Cells were separated from culture supernatants by centrifugation at 4000 x g at 4 °C. Cells
13 were directly resuspended in 1 x SDS sample buffer. Ten-fold of concentrated *P. aeruginosa*
14 culture supernatant was prepared as follows. Proteins of the culture supernatant were
15 precipitated using 6 M trichlo-roacetic acid (TCA) (Sigma) at a final TCA concentration of
16 10%. Protein pellets were washed in 90% acetone, dried and suspended in 1 x SDS sample
17 buffer and incubated at 96 ºC for 20 min and analyzed by SDS-PAGE. *P. aeruginosa*
18 antibacterial activity assay using *E. coli* as target cells was performed as previously described
19 (42). In brief, overnight cultures of indicated *P. aeruginosa* strains were incubated with
20 overnight cultures of equivalent bacterial numbers of *E. coli* containing the plasmid pCR2.1
21 (carrying the *lacZ* gene) in a 1:1 ratio on LB agar for 5 hr at 37 °C. In addition, *P. aeruginosa*
22 and *E. coli* strains grow alone on LB agar for 5 hr at 37 °C served as negative growth controls.
23 Subsequently, patches of bacteria were collected, resuspended in LB broth and dilution series
24 ranging from 10^0 to 10^{-7} were plated in triplicate on LB supplemented with 100 mg/mL
25 5-bromo- 4-chloro-indolyl-β-D-galactopyranoside (X-gal, Invitrogen) allowing for

1 colorimetric detection of *lacZ*-positive *E. coli* survivors. For quantitative analysis of the

2 amount of *E. coli* survivors, the spots were harvested, serially diluted and plated on LB agar

3 plate containing X-gal and appropriate antibiotic to quantify surviving *E. coli* cells by

4 counting colony-forming units (CFUs). Data represent mean ± standard deviation (SD) of all

5 biological replicates.

6 **Yeast two-hybrid assay**

7 The Matchmaker yeast two-hybrid system was used as instructed (Clontech, Mountain View,

8 CA) and as previously described (30). Each of the plasmid pairs were co-transformed into

9 *Saccharomyces cerevisiae* strain AH109. The transformants were selected by their growth on

10 synthetic dextrose (SD) minimal medium lacking tryptophan (Trp) and leucine (Leu) (SD-WL

11 medium). The positive interaction of expressed fusion proteins was then determined by their

12 growth on SD lacking Trp, Leu, adenine (Ade), and histidine (His) (SD-WLHA medium) at

13 30 ^{o}C for at least 2 days.

14 **Total protein extraction from yeast**

15 In brief, to prepare the total protein from yeast (43), overnight grown yeast strains harboring

16 appropriate plasmids were sub-cultured at 28 °C in the same medium for further growth to

17 OD_{600} 0.4-0.6. Yeast cells were harvested, 100 µl of protein extraction buffer containing 0.1%

18 NP-40, 250 mM NaCl, 50 mM Tris-HCl (pH7.5), 5 mM EDTA (pH8.0), 1 mM DTT, 2 x

19 protease inhibitor cocktails (Roche) and 4 mM PMSF, and 50 µL of acid-washed glass beads

20 (Sigma-Aldrich) were added. The cells were broken by vortex at highest speed for 30 seconds,

21 then tubes placed on ice for 30 seconds. The same procedure was repeated for 6 times to make

22 sure cells have been completely broken. The supernatant above the glass beads was collected

23 (the 1st cell extract). Then, 50 µL of protein extraction buffer was added to wash the tube

24 containing broken cells and glass beads by vortexing at highest speed for 30 seconds. The

supernatant above the glass beads was collected again (the 2nd cell extract). The two extracts were mixed together and the final protein extract was centrifuged at 13,000 x rpm for 5 min at 4 °C; the resulting supernatant was collected, and protein concentration was measured. An equal volume of 2 x SDS loading buffer was added to the final protein sample and incubated at 96 ºC for 20 min and analyzed by SDS-PAGE.

Bacterial two-hybrid assay

Bacterial two-hybrid (BTH) assay was performed as previously described (44). In brief, DNA fragments encoding the protein of interest were amplified by PCR, adding appropriate restriction sites into the primers, using *P. aeruginosa* PAK genomic DNA. DNA fragments encoding the proteins or protein domains of interest were cloned into plasmids pKT25 and pUT18C, which each encode for complementary fragments of the adenylate cyclase enzyme, as previously described (44), resulting in constructs expressing N-terminal fusion of the protein of interest with the T25 or T18 subunit of adenylate cyclase. Recombinant pKT25 and pUT18C plasmids were co-transformed into the *E. coli* DHM1 strain, which is devoid of adenylate cyclase, and transformants were spotted onto LB agar plates (Difco) supplemented with 40 mg/mL X-gal, in presence of 100 mg/mL ampicillin, 50 mg/mL kanamycin and 1 mM IPTG. Positive interactions were identified as blue colonies on LB agar plates containing X-gal after 48 hr incubation at 30 °C. The experiments were performed at least in duplicate, and a representative result is shown.

β-galactosidase assay

For quantitative analysis of BTH interactions, β-galactosidase activity of co-transformants scraped from LB agar plates containing X-gal was measured as described previously and activity was calculated in Miller units (45,46).

Statistics analysis

Data represent mean ± standard error (SE) of all biological replicates. Statistics was calculated by one-way ANOVA and Tukey's honestly significance difference (HSD) test (http://astatsa.com/OneWay_Anova_with_TukeyHSD/) and the significant difference was indicated (P <0.01 or P <0.05).

Dephosphorylation and Phos-tag SDS-PAGE analyses

Dephosphorylation analysis by calf intestinal alkaline phosphatase (CIAP) was performed according to the user manual (New England Biolabs, Beverly, MA, USA) with minor modifications as described previously (22). Equal amounts of Ni-NTA resins with purified TssL-His isolated from various *A. tumefaciens* strains were resuspended in 1 X CIAP buffer containing 100 mM NaCl, 50 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 1 mM DTT, and 1 X protease inhibitor cocktail (EDTA-free) with CIAP at 1 unit per μg of protein. The protein samples treated with or without CIAP were incubated at 37 °C for 90 min. An equal volume of 2X SDS loading buffer was added and incubated at 96 ºC for 20 min and analyzed by Phos-tag SDS-PAGE. The Phos-tag SDS-PAGE analysis was performed according to the user manual for Phos-tag Acrylamide AAL-107 (Wako Pure Chemical Industries, Osaka, Japan) with minor modifications as described previously (22). Protein samples were separated on 7% polyacrylamide gels containing 0.35 M Bis-Tris-HCl (pH 6.8), 35 μM Phos-tag Acrylamide AAL-107, and 100 μM $ZnCl_2$, with electrophoresis conducted at 40 mA/gel under a maximum voltage of 90V in a cold room. After electrophoresis, Phos-tag gels were washed with transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) containing 1 mM EDTA for 15 min with gentle shaking followed by a second wash in transfer buffer without EDTA for 15 min. The gels were washed with transfer buffer containing 1% SDS for 15 min before transfer to PVDF membranes with a submarine blotting apparatus.

Protein purification and gel filtration analysis

N-terminal His-tagged TagF 1-214 aa proteins were expressed in *E. coli* BL21 (DE3) cells with the plasmid pET28a(+)-TagF 1-214. *E. coli* cells were grown in LB medium in the presence of kanamycin (20 μg/mL) at 37 °C until the cell density reached to an OD_{600} of 0.6-0.8. The cultures were induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 16-20 hr at 16 °C to induce production of His-tagged TagF 1-214 aa proteins. The cells were harvested by centrifugation followed by snap freezing by liquid nitrogen and stored at -80 °C. Frozen bacterial pellets were resuspended with the lysis buffer (150 mM sodium chloride, 10 mM imidazole, and 50 mM Tris-HCl pH 7.5), and then the cells were lysed by microfluidizer. The cell lysate was clarified by centrifugation at 20,000 rpm for 40 min at 4 °C, and the supernatant was loaded onto a Ni-NTA column (GE Healthcare, USA) pre-equilibrated with the lysis buffer. The column was washed with the washing buffer (150 mM sodium chloride, 80 mM imidazole, and 50 mM Tris-HCl pH 7.5), and the bound protein was eluted by the elution buffer (150 mM sodium chloride, 300 mM imidazole, and 50 mM Tris-HCl pH 7.5). The TagF 1-214 aa proteins were further analyzed by size-exclusion chromatography using a Superdex 75 16 x 60 column through fast protein liquid chromatography (FPLC) system (GE Healthcare). The column was equilibrated with 2 column volumes of buffer containing 50 mM Tris-HCl (pH 7.5), and 150 mM NaCl. To determine molecular weight, a parallel column was run with protein standards. The elution profiles were recorded as absorbance at 280 nm.

Western blot analysis

Western blot analysis was performed as previously described (40) with the primary polyclonal *p*TssL antibody produced in this study and those against proteins (PpkA, TagF-PppA, TssK, Fha, $TssC_{41}$, TssB, TssA, ClpV, Tae, VgrGs, RpoA)(30), TssL (4), TssM (4), Hcp (27), ActC (47), Tde1(29), *P. aeruginosa* Hcp1, TssB1, TagJ1, and Tse3 (44), polyclonal antibodies against His (Sigma-Aldrich), and monoclonal antibodies against HA (Sigma-Aldrich), the

beta subunit of RNA polymerase (RNAP) (Neoclone), c-Myc (Sigma-Aldrich) or Strep (IBA-Life Sciences, Goettingen, Germany), followed by incubation with a secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (chemichem), and detection by use of the Western Lightning System (Perkin Elmer, Boston, MA). Chemiluminescent bands were visualized on X-ray film (GE Healthcare).

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Figure legends

Figure 1 *A. tumefaciens* C58 *t6ss* gene clusters and TagF-pppA domain organization. (A) The *imp* operon (*atu4343* to *atu4330*), *hcp* operon (*atu4344* to *atu4352*), and *vgrG2* in *A. tumefaciens* strain C58 was designated *tss* or *tag* based on nomenclature proposed by Shalom et al. (2007) (48) and specific names derived from Lin *et al.* (30) and Bondage *et al.* (31). **(B)** TagF-PppA domain organization according to information from the NCBI database (http://www.ncbi.nlm.nih.gov/). TagF-PppA is predicted as a cytoplasmic protein (1-471 aa) with an N-terminal conserved TagF (DUF2094) domain (11–219 aa) and a C-terminal PppA (PP2Cc) domain (244-470 aa).

Figure 2 Both TagF and PppA domains can repress type VI secretion and antibacterial activity at post-translational levels in *A. tumefaciens*. (A) Type VI secretion analysis.

1 Western blot analysis of total (T) and secreted (S) proteins isolated from wild-type C58
2 harboring the vector pTrc200 (V) or various overexpressing plasmids grown in AB-MES (pH
3 5.5) liquid culture with specific antibodies. The non-secreted protein ActC and RNA
4 polymerase α subunit RpoA were internal controls. The proteins analyzed and sizes of
5 molecular weight standards are on the left and right, respectively, and with arrowheads when
6 necessary. FL, full length TagF-PppA protein. **(B)** *A. tumefaciens* antibacterial activity assay
7 against *E. coli*. The *A. tumefaciens* wild-type C58 harboring the vector pTrc200 (V) or
8 various overexpressed plasmids or Δ*tssL* mutant harboring the vector pTrc200 (V) was
9 co-cultured on LB agar with *E. coli* strain DH10B cells harboring the plasmid pRL662. **(C)** *A.*
10 *tumefaciens* intraspecies competition *in planta*. The *A. tumefaciens* wild-type C58 harboring
11 the vector pTrc200 (V) or various overexpressed plasmids or Δ*tssL* mutant harboring the
12 vector pTrc200 (V) was used as attacker strain to mix with the target strain Δ*3TIs* harboring
13 pRL662 and infiltrated into *N. benthamiana* leaves. **(B)(C)** Data are mean ± standard
14 deviation (SD) of at least 3 biological replicates. Different letters above the bar indicate
15 statistically different groups of strains [P <0.01 for **(B)**, P<0.05 for **(C)**] based on CFUs of the
16 surviving target cells.

17 **Figure 3 Both TagF and PppA domains repress T6SS activity independently of**
18 **PpkA-mediated TssL phosphorylation pathway in *A. tumefaciens*. (A)** Phos-tag
19 SDS-PAGE analysis to detect the phosphorylation status of TssL-His. Western blot analysis
20 of the same volumes of Ni-NTA resins (10 µL) associated with TssL-His from different
21 strains treated with (+) or without (-) CIAP and examined by a specific antibody against
22 6xHis. Total protein isolated from Δ*tssL* was a negative control. Phos-tag SDS-PAGE
23 revealed the upper band indicating the phosphorylated TssL-His (*p*-TssL-His) and lower band
24 indicating unphosphorylated TssL-His. **(B)(C)** Western blot analysis of the endogenous
25 phosphorylation status of TssL (*p*TssL). Western blot analysis of total proteins isolated from

wild-type C58 or Δ*ppkA* or Δ*tssL* or C58 harboring the vector pTrc200 (V) or various overexpressing plasmids grown in AB-MES (pH 5.5) liquid culture with specific antibodies. The specific antibody for phosphorylated TssL (*p*TssL) was generated against the 15 mer peptide (7-SSWQDLP*p*TVVEITEE-21), with phosphorylated Thr 14 of TssL underlined. RNA polymerase α subunit RpoA was an internal control. The proteins analyzed and molecular weight standards are on the left and right, respectively, and indicated with an arrowhead when necessary. FL, full-length TagF-PppA proteins.

Figure 4 TagF represses T6SS activity independent of the TPP pathway in *A. tumefaciens*. (A) (B) Type VI secretion analysis. Western blot analysis of total (T) and secreted (S) proteins isolated from wild-type C58 harboring the vector pTrc200 (V) or Δ*tssL* harboring the vector pTrc200 (V) or Δ*ppkA*Δ*tagF-pppA* harboring various overexpressing plasmids grown in AB-MES (pH 5.5) liquid culture with specific antibodies. The non-secreted protein ActC and RNA polymerase α subunit RpoA were internal controls. The proteins analyzed and molecular weight standards are on the left and right, respectively, and with an arrowhead when necessary. **(C)** *A. tumefaciens* antibacterial activity assay against *E. coli*. The *A. tumefaciens* wild-type C58 harboring the vector pTrc200 (V) or Δ*tssL* harboring the vector pTrc200 (V) or Δ*ppkA*Δ*tagF-pppA* harboring various overexpressing plasmids was co-cultured on LB agar with *E. coli* strain DH10B cells harboring pRL662. Data are mean ± standard deviation (SD) of at least 3 biological replicates. Different letters above the bar indicate statistically different groups of strains ($P < 0.01$) based on CFUs of the surviving target cells.

Figure 5 TagF directly interacts with Fha of *A. tumefaciens* and *P. aeruginosa*. (A)(B) Yeast two-hybrid protein–protein interaction results. SD-WL medium (SD minimal medium

1 lacking Trp and Leu) was used for the selection of plasmids. SD-WLHA medium (SD
2 minimal medium lacking Trp, Leu, His, and Ade) was used for the auxotrophic selection of
3 bait and prey protein interactions. The positive interaction was determined by growth on
4 SD-WLHA medium at 30 ^{o}C for at least 2 days. The positive control (+) showing interactions
5 of SV40 large T-antigen and murine p53 and negative control (vector) are indicated. **(C)**
6 Bacterial two-hybrid analysis. Various combinations of recombinant pKT25 and pUT18C
7 plasmids harboring *P. aeruginosa* TagF^{Pa} or Fha1^{Pa} proteins were co-transformed into *E. coli*.
8 A graphical representation of the β-galactosidase activity from co-transformants is shown, the
9 plasmid combinations are indicated below, and images of corresponding *E. coli* spots on LB
10 agar plates containing X-gal are displayed at the top. The strength of the interaction was
11 investigated by measuring the β-galactosidase activity of cells. The average activity in Miller
12 units is indicated. Experiments were carried out in duplicate, and data are mean ± standard
13 deviation (SD). Different letters above the bar indicate statistically different groups (P <0.01).
14 T18, empty-vector pUT18C; T25, empty-vector pKT25.

15 **Figure 6 Conserved amino acid residues of TagF are critical for TagF–Fha interaction**
16 **in *A. tumefaciens*. (A)** Amino acid sequence alignment of TagF or TagF domain orthologs
17 from selected bacterial species. Conserved amino acid residues are highlighted in black and
18 those used for mutagenesis are indicated with an asterisk. Sequences were aligned and
19 highlighted by use of ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Part of the
20 aligned result is shown here, and the fully aligned result and full information for bacterial
21 strains and protein accession numbers are in Figure S3A. **(B)** *Agrobacterium* TagF protein is
22 present as a monomer on gel filtration analysis *in vitro*. Purified His-tagged TagF domain
23 (1-214 aa) was analyzed by SDS-PAGE. The proteins analyzed and molecular weight
24 standards are on the right and left, respectively. His-tagged TagF proteins were further
25 analyzed by use of Superdex 75 16 x 60 column and the elution profiles were recorded as

1 absorbance at 280 nm showing His-tagged TagF elutes as a single peak (~26 kDa monomer).
2 **(C)** Relative positions of the conserved amino acids residues in *P. aeruginosa* $TagF^{Pa}$ protein
3 revealed as a monomer crystal structural information according to the X-ray crystal structure
4 of *P. aeruginosa* TagF monomer (34). The corresponding conserved amino acid residues of *A.*
5 *tumefaciens* TagF are indicated in parenthesis. **(D)** Yeast two-hybrid protein–protein
6 interaction results with Fha and various TagF proteins. SD-WL medium (SD minimal
7 medium lacking Trp and Leu) was used for selecting plasmids. SD-WLHA medium (SD
8 minimal medium lacking Trp, Leu, His, and Ade) was used for the auxotrophic selection of
9 bait and prey protein interactions. The positive interaction was determined by growth on
10 SD-WLHA medium at 30 ^{o}C for at least 2 days. The positive control (+) showing interactions
11 of SV40 large T-antigen and murine p53 and negative control (vector) are indicated.

12 **Figure 7 Conserved amino acid residues of TagF are required for repressing type VI**
13 **activity in *A. tumefaciens*. (A)** Western blot analysis of total (T) and secreted (S) proteins
14 isolated from wild-type C58 harboring the vector pTrc200 (V) or various TagF-Strep
15 overexpressed plasmids grown in AB-MES (pH 5.5) liquid culture with specific antibodies.
16 The non-secreted protein ActC and RNA polymerase α subunit RpoA were internal controls.
17 The proteins analyzed and molecular weight standards are on the left and right, respectively.
18 **(B)** *A. tumefaciens* antibacterial activity assay against *E. coli*. The *A. tumefaciens* wild-type
19 C58 or Δ*tssL* or chromosomally encoded *tagF-pppA* variants, including *tagF-pppA* with
20 substitutions in *tagF* domain ($tagF^{GK}$*-pppA*, $tagF^{DW}$*-pppA*, and $tagF^{SDR}$*-pppA*) was co-cultured
21 on LB agar with *E. coli* strain DH10B cells harboring the plasmid pRL662. Data are mean ±
22 standard deviation (SD) of at least 3 biological replicates. Different letters above the bar
23 indicate statistically different groups of strains (P <0.05) based on CFUs of the surviving
24 target cells.

1 **Figure 8 Conserved amino acid residues of TagF^{Pa} critical for TagF^{Pa}–Fha1^{Pa} interaction**
2 **are required for repressing H1-T6SS activity in *P. aeruginosa*. (A)** Yeast two-hybrid
3 protein–protein interaction results with *P. aeruginosa* Fha1 and various *P. aeruginosa* TagF
4 proteins. SD-WL medium (SD minimal medium lacking Trp and Leu) was used for selecting
5 plasmids. SD-WLHA medium (SD minimal medium lacking Trp, Leu, His, and Ade) was
6 used for auxotrophic selection of bait and prey protein interactions. The positive interaction
7 was determined by growth on SD-WLHA medium at 30 °C for at least 2 days. The positive
8 control (+) showing interactions of SV40 large T-antigen and murine p53 and negative
9 control (vector) are indicated. **(B)** *P. aeruginosa* H1-T6SS secretion analysis. Western blot
10 analysis of total (T) or secreted (S) proteins isolated from *P. aeruginosa* PAKΔ*retS*
11 (H1-T6SS-induced) harboring the vector pRL662 (V) or PAKΔ*retS* harboring various
12 overexpressed plasmids grown in tryptone soy broth (TSB) with specific antibodies. The
13 non-secreted RNA polymerase β subunit (RNAP) was an internal control. The proteins
14 analyzed and molecular weight standards are on the left and right, respectively, and with an
15 arrowhead when necessary. **(C)** *P. aeruginosa* H1-T6SS–mediated antibacterial assay against
16 *E. coli*. Overnight cultures of *P. aeruginosa* PAKΔ*retS* or PAKΔ*retS*Δ*tssB1* (T6SS-defective
17 strain) harboring the vector pRL662 (V) or various *tagF-Strep* overexpressing plasmids were
18 mixed with equivalent numbers of *E. coli* DH5α carrying a plasmid (pCR2.1) expressing
19 β-galactosidase. Data are mean ± standard deviation (SD) of at least 3 biological replicates.
20 Different letters above the bar indicate statistically different groups of strains (P <0.05) based
21 on CFUs of the surviving target cells. **(D)** Presence of Fha1^{Pa} increases the stability of TagF^{Pa}
22 protein in *P. aeruginosa*. Western blot analysis of total (T) proteins isolated from *P.*
23 *aeruginosa* PAKΔ*retS* (H1-T6SS-induced) or PAKΔ*retS*Δ*H1* (deletion of *retS* and *H1-T6SS*
24 cluster) harboring various plasmid combinations grown in TSB with specific antibodies. All
25 protein samples were analyzed by SDS-PAGE followed by Coomassie blue staining (CBR)

and served as an internal control. The proteins analyzed and molecular weight standards are on the left and right, respectively, and with arrowheads when necessary.

Figure 9 Proposed models of TPP activation and TagF-mediated post-translational repression of type VI secretion in *A. tumefaciens* and *P. aeruginosa*.

Proposed models of TPP activation (T6SS ON) and TagF-mediated repression (T6SS OFF) in *A. tumefaciens* (upper panel) and *P. aeruginosa* (lower) are illustrated. Key activation or repression events are summarized at the bottom of each model. Protein names are indicated in or near the designated molecules. IM: inner membrane, OM: outer membrane. Detailed description of proposed models is in the text.

Supporting Information Legends

Information S1

Figure S1 Type VI secretion assay in Δ*tssL* harboring two plasmids. Western blot analysis of secreted (S) **(A)** and total (T) **(B)** proteins isolated from Δ*tssL* (pTssL-His) harboring the vector pTrc200 (V) or *tagF-pppA*–overexpressing plasmid (pTrc-TagF-PppA) or *tagF*–overexpressing plasmid (pTrc-TagF) or *tagF-Strep*–overexpressing plasmid (pTrc-TagF-Strep) or *pppA*–overexpressing plasmid (pTrc-PppA) grown in AB-MES (pH 5.5) liquid culture with specific antibodies. The non-secreted protein ActC and RNA polymerase α subunit RpoA were internal controls. The proteins analyzed and molecular weight standards are on the left and right, respectively, and indicated with an arrowhead when necessary. FL, full-length TagF-PppA proteins.

Figure S2 Yeast two-hybrid protein–protein interaction studies. Yeast two-hybrid protein–protein interaction results with **(A)** *P. aeruginosa* TagF ($TagF^{Pa}$) and Fha1 ($Fha1^{Pa}$)

proteins and **(B)** *A. tumefaciens* TagF proteins. SD-WL medium (SD minimal medium lacking Trp and Leu) was used for selecting plasmids. SD-WLHA medium (SD minimal medium lacking Trp, Leu, His, and Ade) was used for auxotrophic selection of bait and prey protein interactions. The positive interaction was determined by growth on SD-WLHA medium at 30 ^{o}C for at least 2 days. The positive control (+) showing interactions of SV40 large T-antigen and murine p53 and negative control (vector) are indicated.

Figure S3 Amino acid sequence alignment of TagF orthologs, and western blot analysis of total proteins from yeast with various plasmid combinations. (A) Amino acid sequence alignment of the TagF or TagF domain orthologs from *A. tumefaciens* (TagF-PppA/Atu4331, accession: NP_356324.2), *P. aeruginosa* (TagF/PA0076, accession: NP_248766.1), *Nitrococcus mobilis* (NB231_12224, accession: ZP_01126757.1), *Burkholderia thailandensis* (Hypothetical protein BTH_I2955, accession: YP_443462.1), and *Pseudomonas syringae* (Hypothetical protein PSPPH_0124, accession: YP_272434.1). Conserved amino acid residues are highlighted in black and marked below, and G22, K23, D44, W46, S93, D95, R99, F141 and D142 used for mutagenesis are indicated with an asterisk. The relative positions of these conserved amino acid residues in *P. aeruginosa* $TagF^{Pa}$ are respectively indicated in parentheses. The residues required for dimer formation in *P. aeruginosa* TagF (V105, L169, L172, A173, and L195 in $TagF^{Pa}$) (34) are indicated with a down arrow. Sequences were aligned and highlighted by use of ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Part of the aligned result is shown in Figure 6A. **(B)** According to Figure 6D. Western blot analysis of total (T) proteins isolated from yeast with various plasmid combinations with specific antibodies. The AD vector expressing SV40 large T-antigen (T) or *A. tumefaciens* Fha proteins tagged with HA, and BD vector expressing murine p53 (53) or various *A. tumefaciens* TagF (TagF, $TagF^{GK}$, $TagF^{DF}$, $TagF^{DW}$, $TagF^{SDR}$, and $TagF^{FD}$) proteins tagged with Myc. **(C)** According to Figure 8A. Western blot analysis of

total (T) proteins isolated from yeast with various plasmid combinations with specific antibodies. The AD vector expressing SV40 large T-antigen (T) or *P. aeruginosa* Fha1 ($\mathrm{Fha1}^{\mathrm{Pa}}$) proteins tagged with HA, and BD vector–expressing murine p53 (53) or various *P. aeruginosa* TagF ($\mathrm{TagF}^{\mathrm{Pa}}$, $\mathrm{TagF}^{\mathrm{Pa\text{-}GK}}$, and $\mathrm{TagF}^{\mathrm{Pa\text{-}SDR}}$) proteins tagged with Myc. All protein samples were analyzed by SDS-PAGE followed by Coomassie blue staining (CBR) and served as an internal control. The proteins analyzed and molecular weight standards are on the left and right, respectively, and indicated with an arrowhead when necessary.

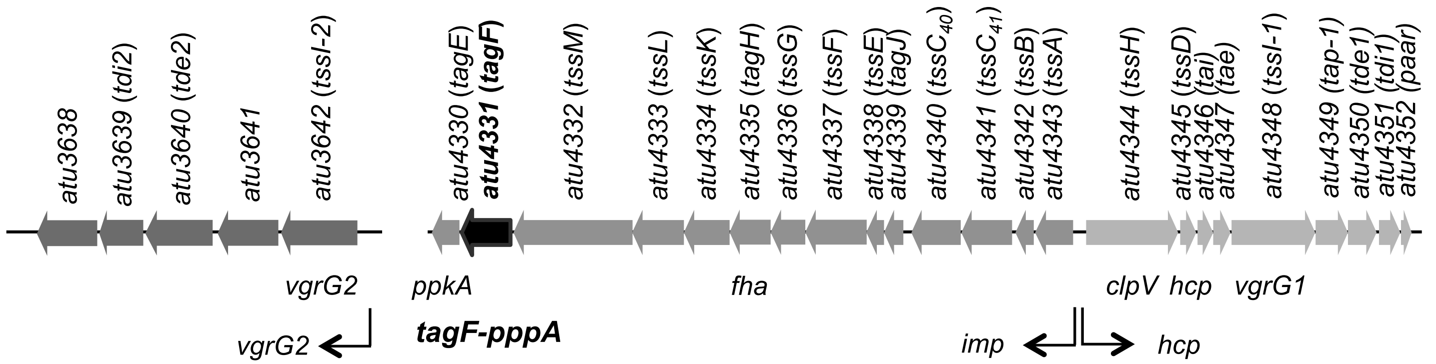
Figure S4 Type VI secretion analysis. Western blot analysis of total (T) and secreted (S) proteins isolated from wild-type C58, Δ*tssL,* Δ*tagF-pppA,* or chromosomally encoded *tagF-pppA* variants, including *tagF-pppA* with substitutions of *tagF* domain ($tagF^{GK}$*-pppA*, $tagF^{DW}$*-pppA*, and $tagF^{SDR}$*-pppA*) grown in AB-MES (pH 5.5) liquid culture with specific antibodies. The non-secreted protein ActC and RNA polymerase α subunit RpoA were internal controls. The proteins analyzed and sizes of molecular weight standards are on the left and right, respectively, and with arrow when necessary.

Table S1. Bacterial strains and plasmids.

Table S2. Primer information.

Fig. 1

(A)



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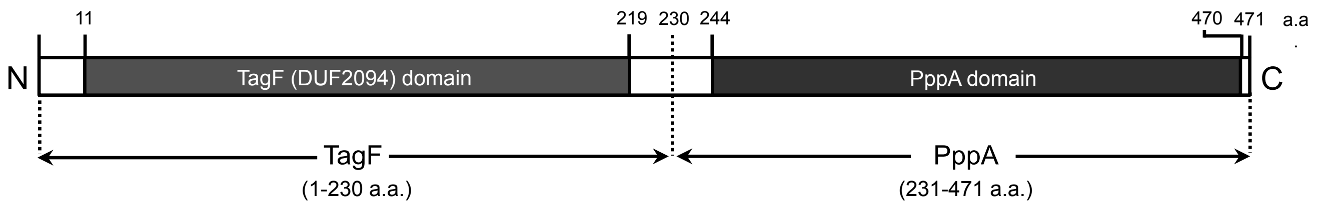
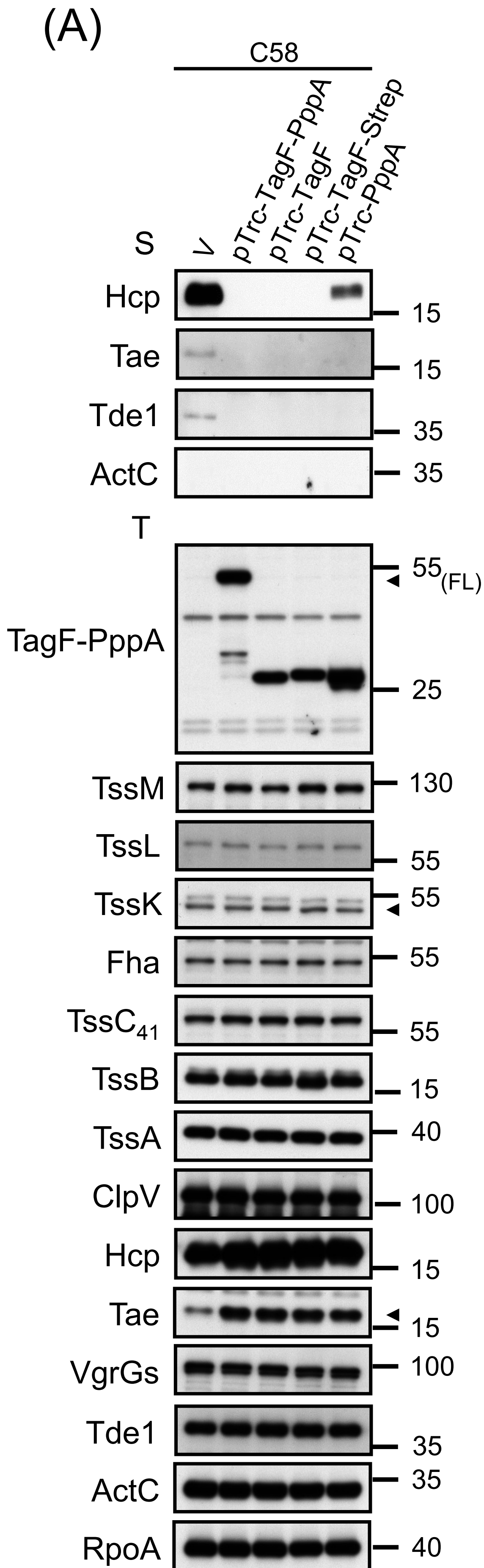
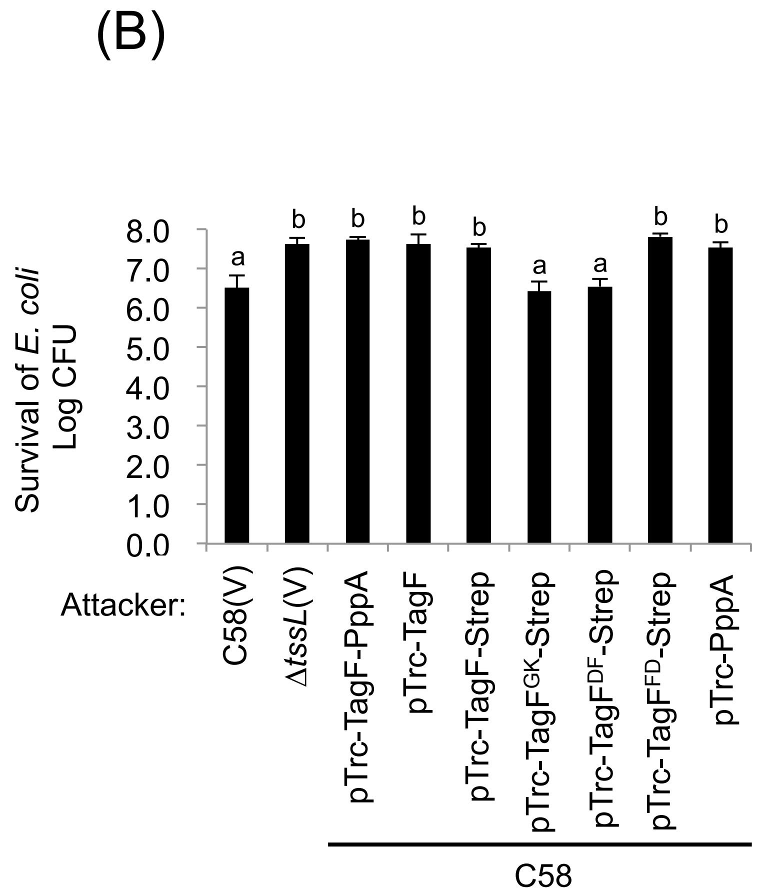


Fig. 2





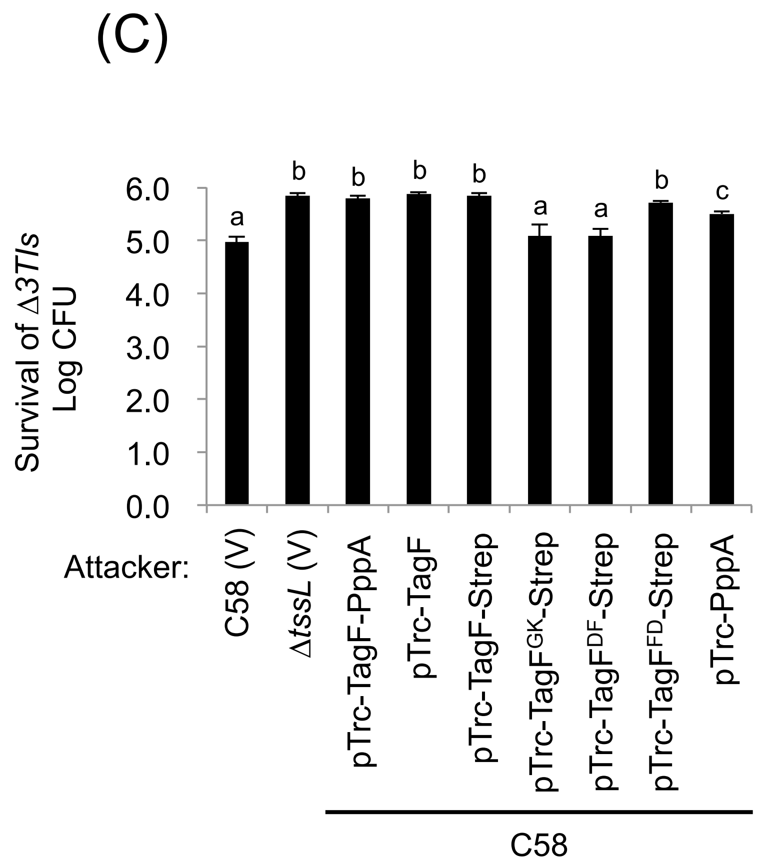
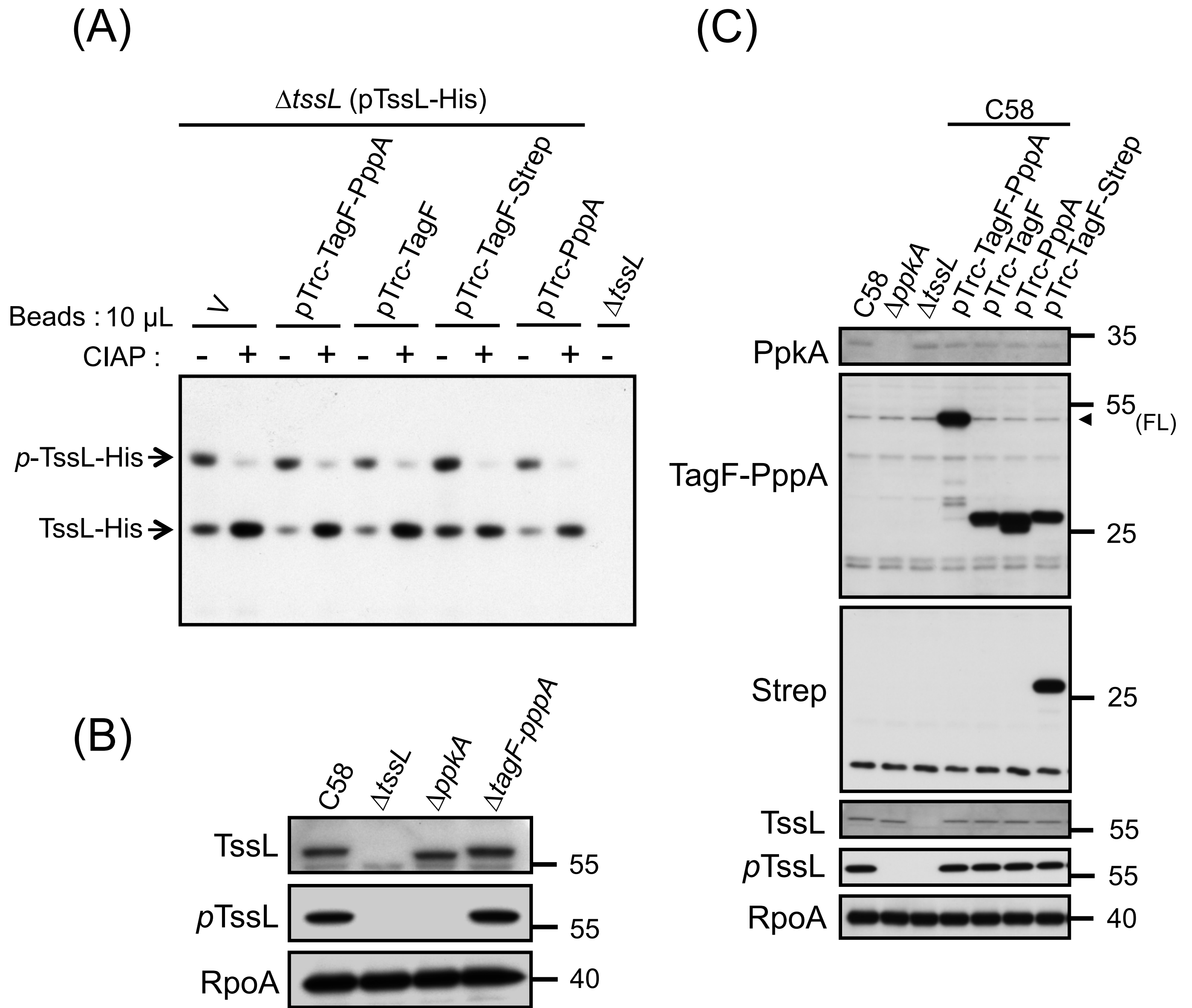


Fig. 3



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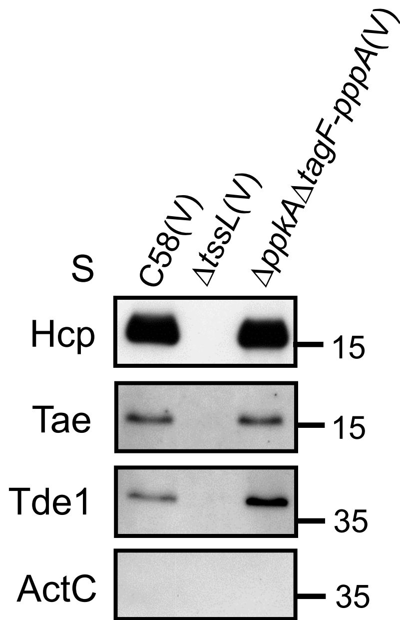
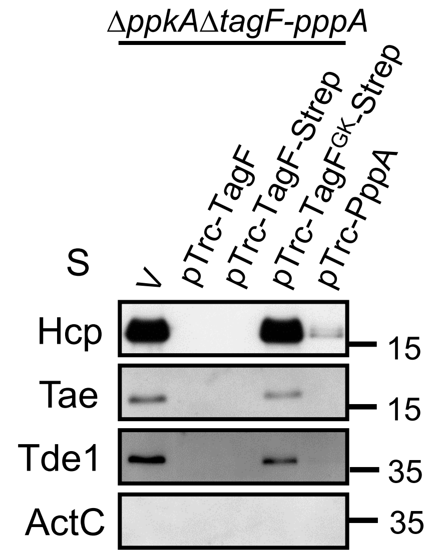
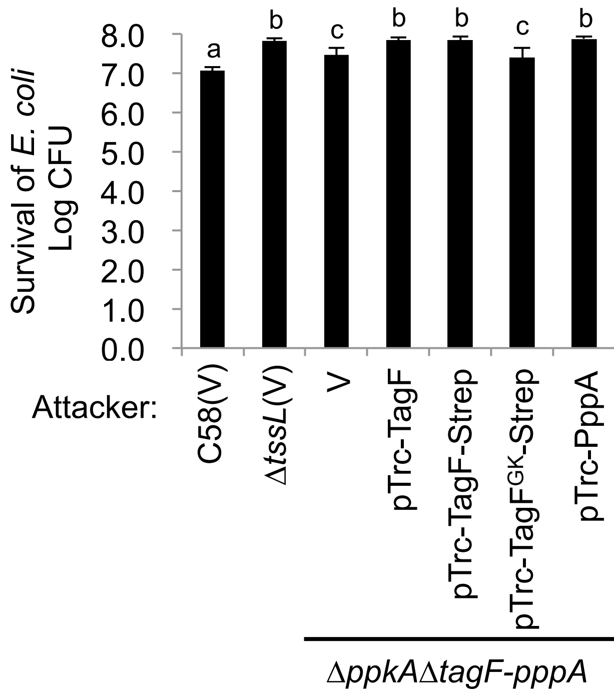






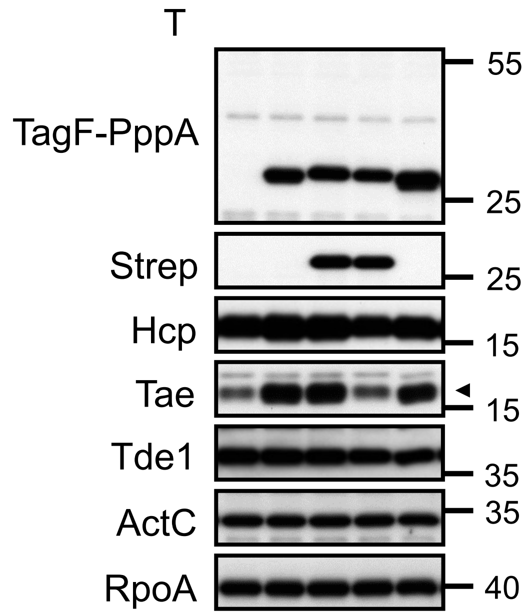
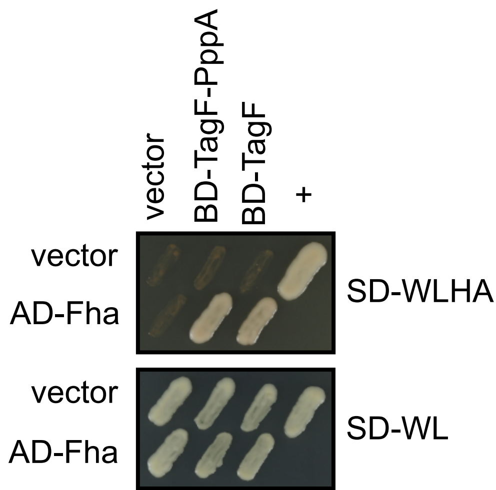


Fig. 5

(A)



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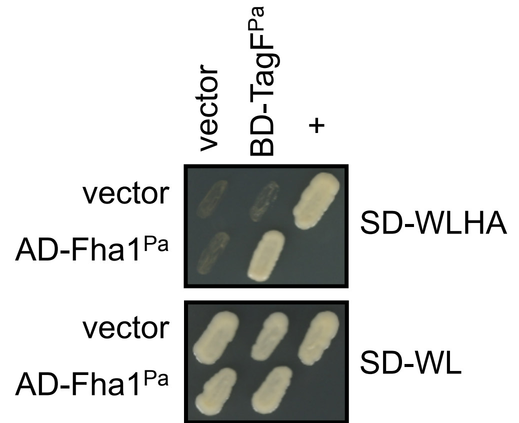

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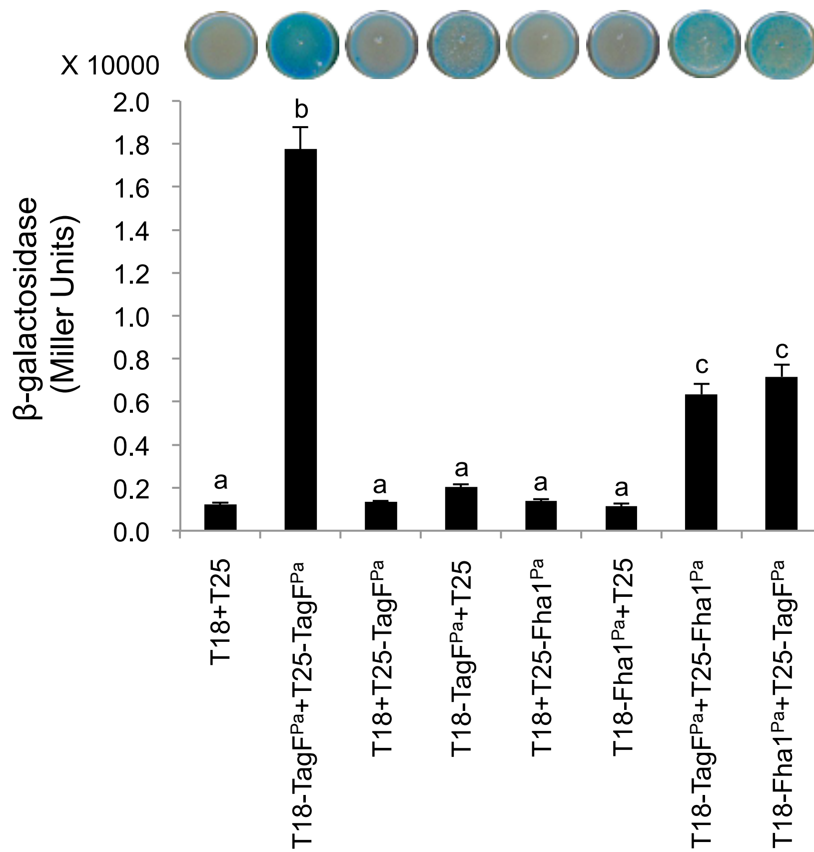

Fig. 6

(A)

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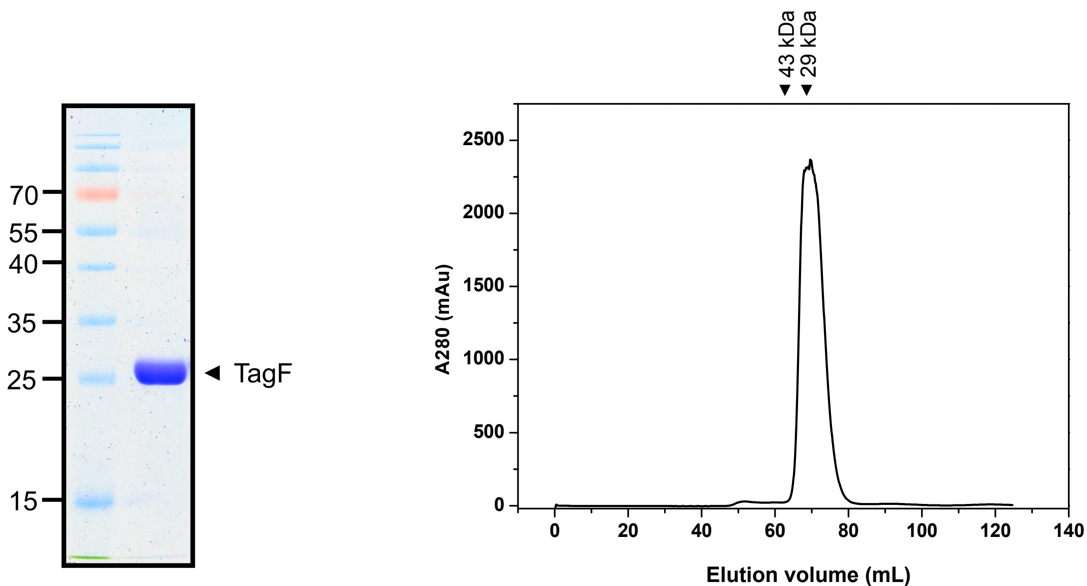
                                                        G22 K23      D29 F30               D44 W46
                                                        **           **                    *  *
A. tumefaciens TagF domain 1-230 a.a.     : MADQASRSTQTAAESDRIGFFGKVPSHGDFISDGLERELIATFDDWMRSGMHACADMFAGRWTEIFSSSPPI :
P. aeruginosa TagF 226 a.a.               : --------------MNSVGFYGKLAGRGDFVSRGLPNTFVEPWDAWLASGMRASQDELGAAWLDAYLTSPLW :
N. mobilis TagF domain 1-160 a.a.         : -----------MVGVGALGFYGKLPSHGDFVRRRVPHEFLTVWDAWLQRVLHASRQRLNDQWLATYLTSPIW :
B. thailandensis TagF domain 1-160 a.a.   : ---------MTQTVQAQIAYFGKIPSRGDFVKSAHNPQLLQTLDHWIARAMELLAD--DPRWKIVYESAKPM :
P. syringae TagF domain 1-230 a.a.        : ----------------MIGCFGKVPASADFVSLHGASDDVCEFDAWLQGALADMQQ--RDDWRTLFDQLPVC :
                                                              GK     DF              D W              W

                                                                         S93 D95   R99                                            F141 D142
                                                                         *   *     *                                              **
A. tumefaciens TagF domain 1-230 a.a.     : RFIVESGIWGNCAYAGVLVPSADRVGRKYPLAIIAQLNGF--RRHPRTLYLDDTWFMAVEALAETSMTGDFD :
P. aeruginosa TagF 226 a.a.               : RFAIAPGLLGGEAVTGVVMPSIDRVGRYFPLTVACLLPAN--ADLGGLVGGDDGWFEQVESLLLSTLEPEAE :
N. mobilis TagF domain 1-160 a.a.         : RFAIDPPVCGRLAYTGILMPSVDAIGRYFPLSFVMPLPVG--MSLPNLAVENADWFAQMERLALSVLEQDVE :
B. thailandensis TagF domain 1-160 a.a.   : HFAFLGSKS-RLAIAGHMIASHDASNRRFPFLAATALEVE--RPLT-FLARSPLAFARLWSRAAAQMQSLLG :
P. syringae TagF domain 1-230 a.a.        : FFSYRARSG--NWLVGGLISSRDSSARRYPFFIFQTVKSSDAGVFVNPFTLSELFAGQIKPLLHMAAQGEGT :
                                             F             G    S D   R  P
```

(B)



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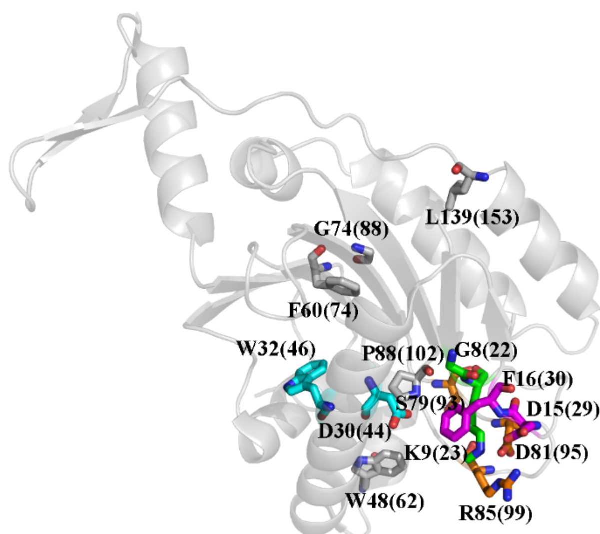

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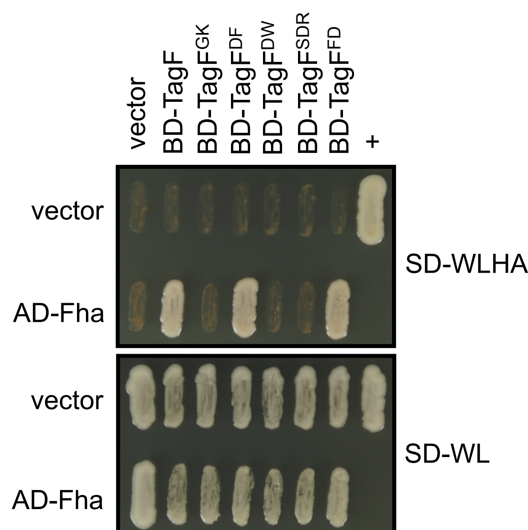


Fig. 7

(A)

C58

S

V
pTrc-TagF-Strep
pTrc-TagF^{GK}-Strep
pTrc-TagF^{DF}-Strep
pTrc-TagF^{DW}-Strep
pTrc-pTagF^{SDR}-Strep

Hcp 15
Tae 15
Tde1 35
ActC 35

T

TagF-PppA 25
Strep 25
TssL 55
ClpV 100
Hcp 15
Tae 15
Tde1 35
ActC 35
RpoA 40

C58

pTrc-TagF-Strep
pTrc-TagF^{GK}-Strep
pTrc-TagF^{FD}-Strep

T S T S T S

Hcp 15
Tae 15
Tde1 35
ActC 35

C58

T

pTrc-TagF-Strep
pTrc-TagF^{GK}-Strep
pTrc-TagF^{FD}-Strep

TagF-PppA 25
Strep 25
ActC 35
RpoA 40

(B)

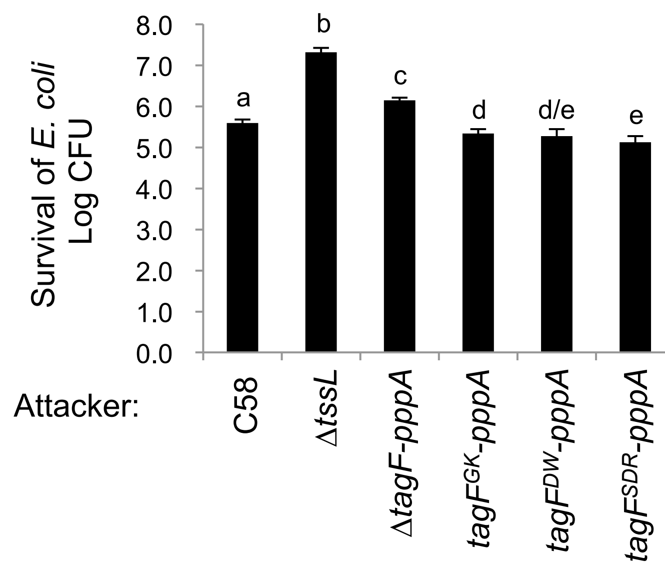


Fig. 8

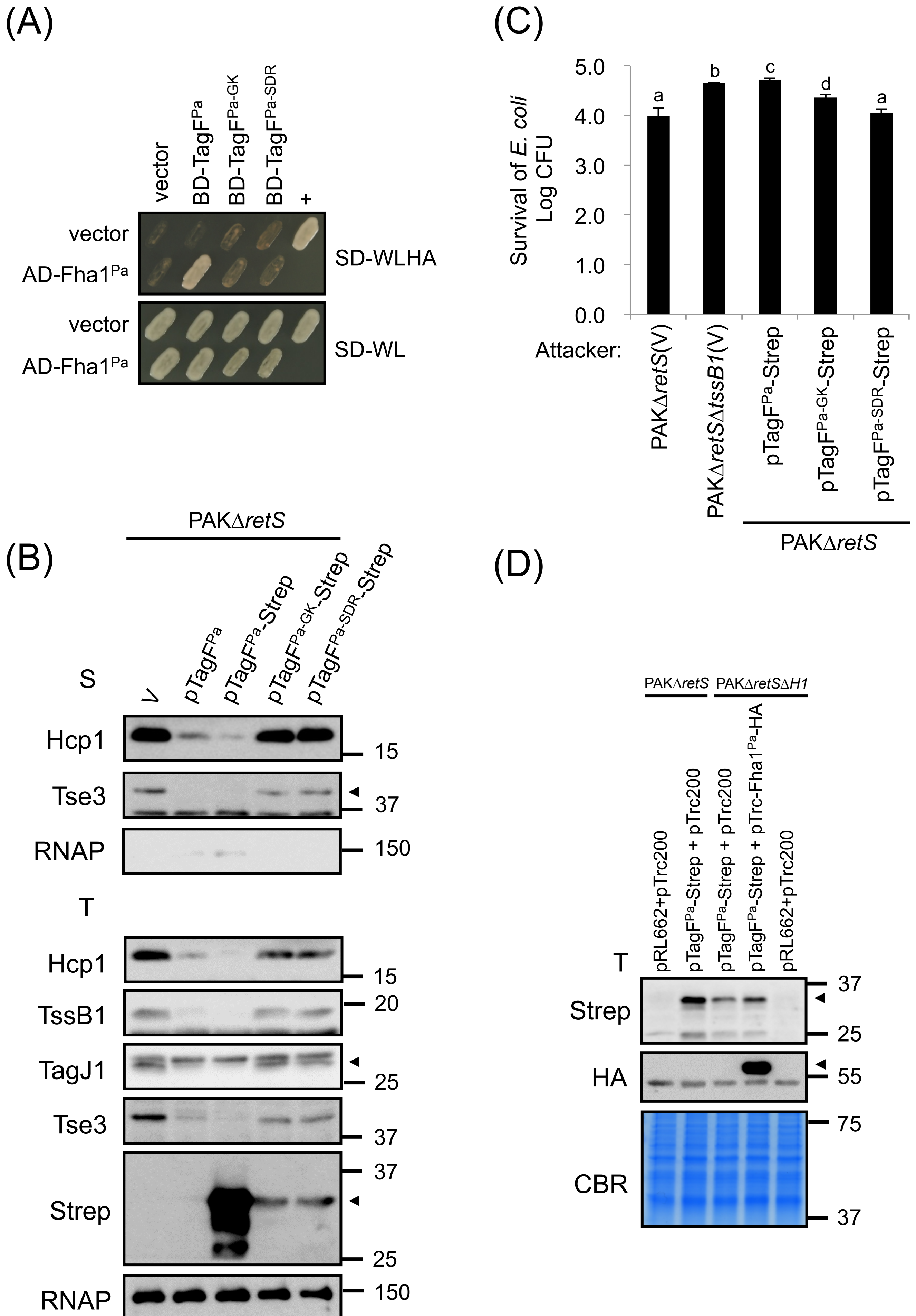


Fig. 9

T6SS ON *A. tumefaciens* T6SS OFF

PAAR
VgrG
OM
Hcp
Periplasm
IM
TssM TssL Base plate
P
PpKA
TssBC
Fha
ClpV
TagF-PppA

Unknown signal

TagF-PppA

TssM TssL Base plate
P
PpKA
PAAR
VgrG
Hcp
Fha
TagF-PppA
TssBC
effector

TPP-dependent activation
-TssL phosphorylation
-Fha recruitment

TagF-PppA-mediated repression
-TagF-PppA binding to Fha
-No Fha recruitment

T6SS ON *P. aeruginosa* T6SS OFF

PAAR
VgrG
OM
Hcp
Periplasm
IM
TssM TssL Base plate
Fha
TssBC
PpKA
P
ClpV
PppA
TagF

Unknown signal

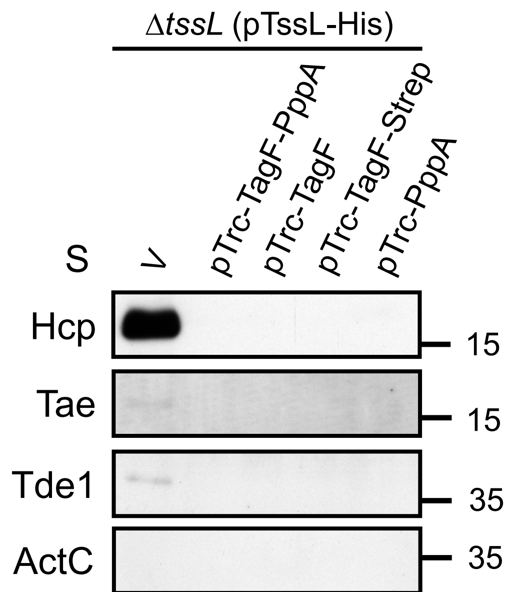
TagF

TssM TssL Base plate
PpKA
PppA
P
Fha
TagF
Hcp
TagJ
TssBC
effector

TPP-dependent activation
-Fha phosphorylation
-Fha recruitment

TagF-mediated repression
-TagF binding to Fha
-No Fha recruitment
-T6SS components unstable

Fig. S1



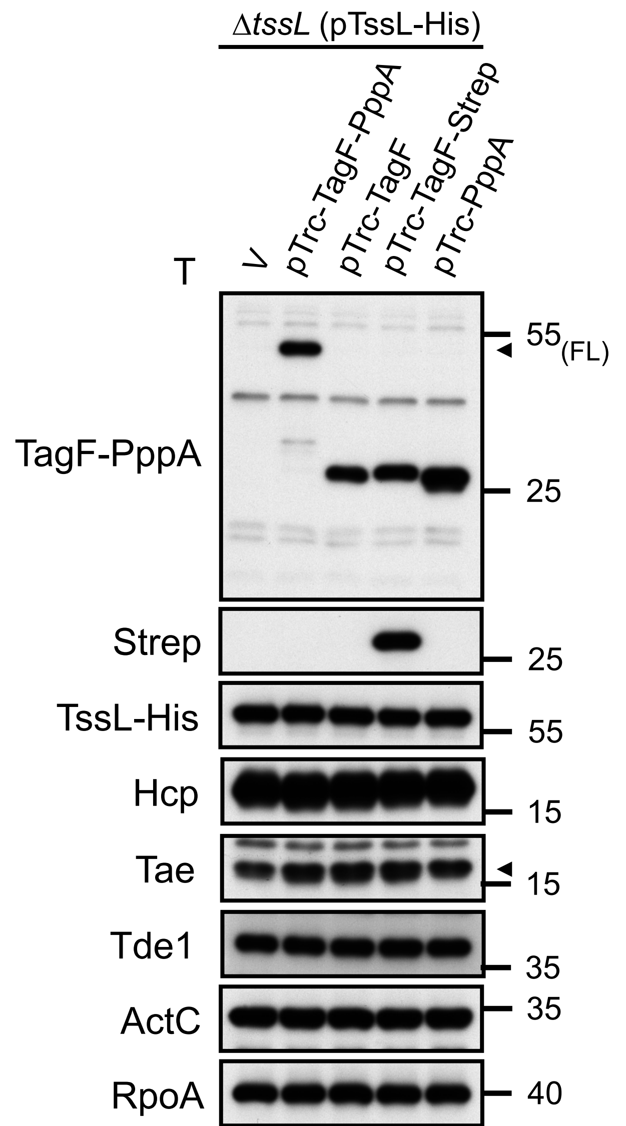
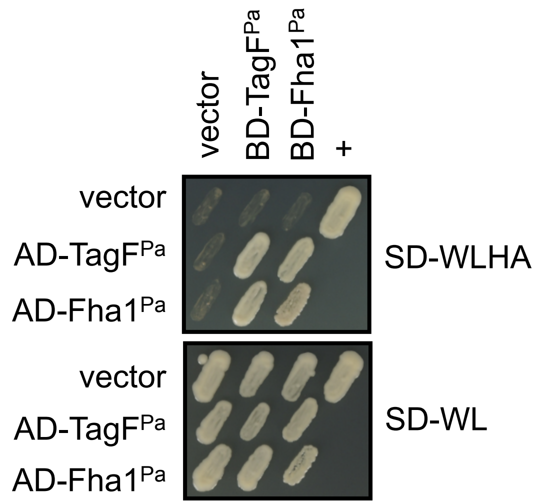


Fig. S2



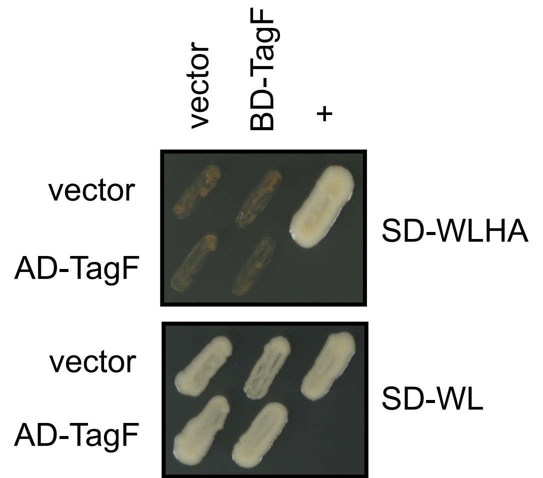


Fig. S3

(A)

Annotations above block 1: * G22 (G8), * K23 (K9), * D29 (D15), * F30 (F16), * D44 (D30), * W46 (W32), (W48)

```
A. tumefaciens TagF domain 1-230 a.a.  : MADQASRSTQTAAESDRIGFFGKVPSHGDFISDGLERELIATFDDWMRSGMHACADMFAGRWTEIFSSSPPI : 72
P. aeruginosa TagF 226 a.a.            : --------------MNSVGFYGKLAGRGDFVSRGLPNTFVEPWDAWLASGMRASQDELGAAWLDAYLTSPLW : 58
N. mobilis TagF domain 1-160 a.a.      : ------------MVGVALGFYGKLPSHGDFVRRRVPHEFLTVWDAWLQRVLHASRQRLNDQWLATYLTSPIW : 61
B. thailandensis TagF domain 1-160 a.a.: ---------MTQTVQAQIAYFGKIPSRGDFVKSAHNPQLLQTLDHWIARAMELLAD--DPRWKIVYESAKPM : 61
P. syringae TagF domain 1-230 a.a.     : ----------------MIGCFGKVPASADFVSLHGASDDVCEFDAWLQGALADMQQ--RDDWRTLFDQLPVC : 54
                                          GK     DF              D W               W
```

Annotations above block 2: (F60), (G74), * S93 (S79), * D95 (D81), * R99 (R85), (P88), ▼ (V105), * F141, * D142

```
A. tumefaciens TagF domain 1-230 a.a.  : RFIVESGIWGNCAYAGVLVPSADRVGRKYPLAIIAQLNGF--RRHPRTLYLDDTWFMAVEALAETSMTGDFD : 142
P. aeruginosa TagF 226 a.a.            : RFAIAPGLLGGEAVTGVVMPSIDRVGRYFPLTVACLLPAN--ADLGGLVGGDDGWFEQVESLLLSTLEPEAE : 128
N. mobilis TagF domain 1-160 a.a.      : RFAIDPPVCGRLAYTGILMPSVDAIGRYFPLSFVMPLPVG--MSLPNLAVENADWFAQMERLALSVLEQDVE : 131
B. thailandensis TagF domain 1-160 a.a.: HFAFLGSKS-RLAIAGHMIASHDASNRRFPFLAATALEVE--RPLT-FLARSPLAFARLWSRAAAQMQSLLG : 129
P. syringae TagF domain 1-230 a.a.     : FFSYRARSG--NWLVGGLISSRDSSARRYPFFIFQTVKSSDAGVFVNPFTLSELFAGQIKPLLHMAAQGEGT : 124
                                          F             G    S D   R  P
```

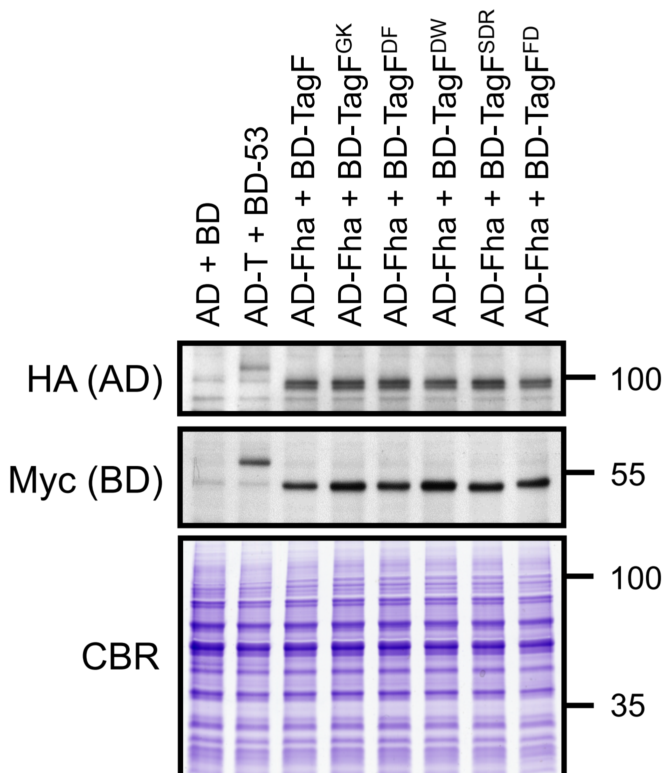
Annotations above block 3: (L139), ▼ (L169), ▼ (L172), ▼ (A173)

```
A. tumefaciens TagF domain 1-230 a.a.  : VS--RFNASIKKLRLPKPRDEDDAPEMVRGRAPTSLWWHIDPVSRRARGLKFDGKPRASDFLRLFSDMTGKG : 212
P. aeruginosa TagF 226 a.a.            : V--EAFEQAVAQLPAPPCGPRIEQSLISG----NLLRSEAVTPAQRLAALAQHACDGASHWWGRGSARISAG : 194
N. mobilis TagF domain 1-160 a.a.      : FRLDDFDRQVAALG---------REFMPR----TLLQDEWQP------------------------------ : 160
B. thailandensis TagF domain 1-160 a.a.: PQ--EPPGALQALG-----------------------DTQVPIDTGGPGSAHDGTF---------------- : 160
P. syringae TagF domain 1-230 a.a.     : SVLFERIRALRPLQGQD--FELFRRVHEKFLVNFTLRDIATSLESSYPEFISNAVLTRLQALGRPSYRAPIG : 194
                                                     L
```

Annotation above block 4: ▼ (L195)

```
A. tumefaciens TagF domain 1-230 a.a.  : DEDGSRAAPAPATVQEKT------------------ : 230
P. aeruginosa TagF 226 a.a.            : LMRYQGLPPAPAFGRFLTGEGEVIPLFPGIPG---- : 226
N. mobilis TagF domain 1-160 a.a.      : ------------------------------------ :   -
B. thailandensis TagF domain 1-160 a.a.: ------------------------------------ :   -
P. syringae TagF domain 1-230 a.a.     : ISLPLPAERGLKNPTADLWVNWLTRIDPNKAVPQIS : 230
```

(B)



(C)

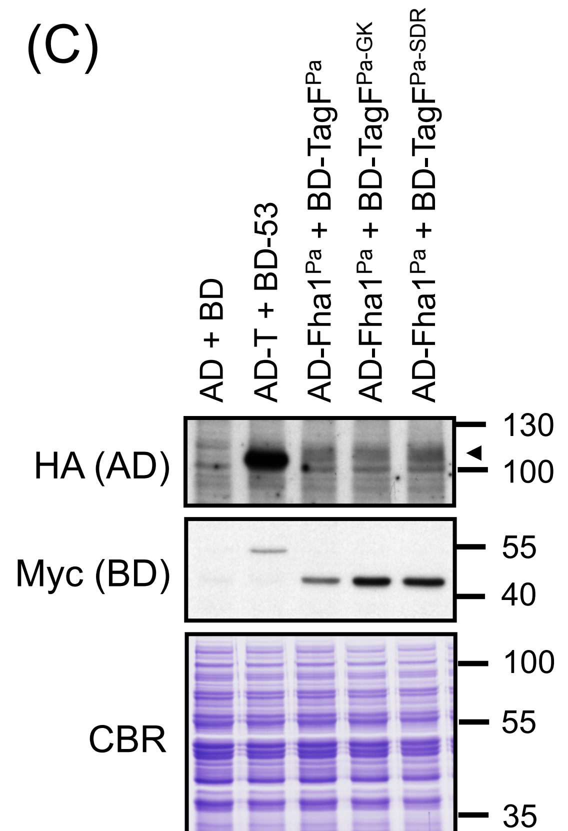
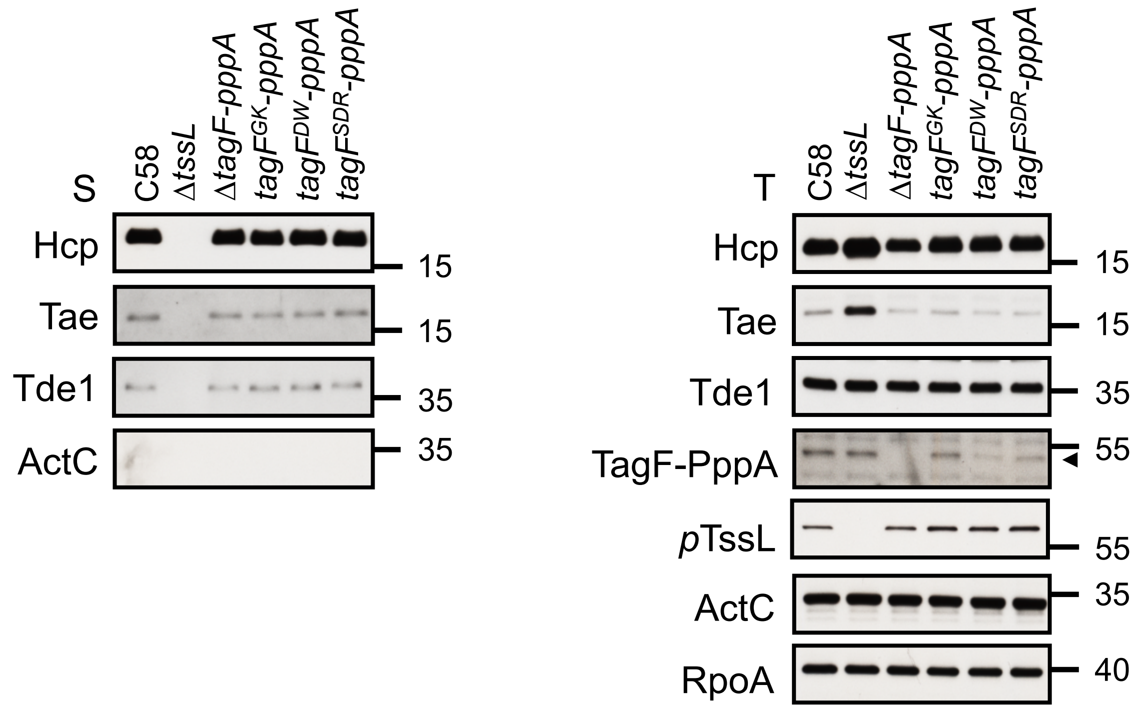


Fig. S4



1 **Supporting Information**

2 **TagF-mediated repression of bacterial type VI secretion systems involves a direct**

3 **interaction with the cytoplasmic protein Fha**

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6

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12

13

14 Running title: TagF-mediated T6SS repression

15 Key words: Type VI secretion system, antibacterial activity, post-translational regulation,

16 TagF, PppA, Fha, *Agrobacterium tumefaciens, Pseudomonas aeruginosa*, protein-protein

17 interaction

18

19

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Supporting Information Legends

Information S1

The growth conditions. *A. tumefaciens* was grown at 25 °C in 523 (1), whereas LB (2) were routinely used for *E. coli* and *P. aeruginosa* strains at 37 °C unless indicated. The plasmids were maintained by the addition of 50 μg/mL gentamycin (Gm) and 200 μg/mL spectinomycin (Sp) for *A. tumefaciens* and 100 μg/mL ampicillin (Ap), 100 μg/mL spectinomycin (Sp), 20 μg/mL kanamycin (Km), and 50 μg/mL Gm for *E. coli*, and 50 μg/mL Gm, and 2000 μg/mL Sp for *P. aeruginosa.* Growth conditions are as previously described (3,4).

Plasmid construction and generation of in-frame deletion mutants. Plasmid pJQ200KS-Δ*ppkA*Δ*tagF-pppA* (Supplementary Table S1) was created by ligating the *Xba*I/*Bam*HI-digested PCR product 1 (~500 bp DNA fragment upstream of *tagF-pppA* gene) and the *BamH*I/*Xma*I-digested PCR product 2 (~500 bp DNA fragment downstream of *ppkA* gene) into *Xba*I/*Xma*I sites of pJQ200KS (5) and used to generate the *ppkA* and t*agF-pppA* genes deletion mutant (Supplementary Tables S1 and S2). The resulting strain was confirmed by PCR and designated as EML4307 (Δ*ppkA*Δ*tagF-pppA*).

To construct the plasmids for expressing proteins in *A. tumefaciens*, each DNA fragment containing the ribosomal-binding sequence (RBS) and ORF (with stop codon) of *tagF-pppA*, *tagF*, *tagF-Strep*, *tagF*^{GK}*-Strep*, *tagF*^{DF}*-Strep*, *tagF*^{DW}*-Strep*, *tagF*^{SDR}*-Strep*, *tagF*^{FD}*-Strep*, and *pppA* were PCR-amplified with primers described in Supplementary Table S2 and respectively cloned into pTrc200 (6) with appropriate enzyme sites to create the plasmids pTrc-TagF-PppA, pTrc-TagF, pTrc-TagF-Strep, pTrc-TagF^{GK}-Strep, pTrc-TagF^{DF}-Strep, pTrc-TagF^{DW}-Strep, pTrc-TagF^{SDR}-Strep, pTrc-TagF^{FD}-Strep, and pTrc-PppA.

To construct the plasmids for expressing proteins in *P. aeruginosa*, the gene of interest containing its RBS and ORF was cloned to be driven by a *lac* promoter on the broad host range vector pRL662 (7). The PCR products of *P. aeruginosa tagF*, *tagF-Strep*, *tagF*^{GK}*-Strep*, and *tagF*^{SDR}*-Strep* were amplified with primers described in Supplementary Table S2 and respectively digested by *Xho*I/*Xba*I, and cloned into the same sites of pRL662, which resulted in the plasmids pTagF^{Pa}, pTagF^{Pa}-Strep, pTagF$^{Pa\text{-}GK}$-Strep, and pTagF$^{Pa\text{-}SDR}$-Strep. The PCR product of *P. aeruginosa fha1-HA* was amplified with primers described in Supplementary Table S2 and cloned into pTrc200 with appropriate enzyme sites, which resulted in the plasmid pTrc-Fha1^{Pa}-HA.

To construct the plasmid for protein expression in *E. coli*, the DNA fragment containing *tagF* 1-214 was PCR-amplified with primers described in Supplementary Table S2 and cloned

into pET28a(+) to create the plasmid pET28a(+)-TagF 1-214.

For the constructs used for yeast two-hybrid, the *fha*, *tagF-pppA*, *tagF*, *tagF*^{GK}, *tagF*^{DF}, *tagF*^{DW}, *tagF*^{SDR}, *tagF*^{FD}, *tagF*^{Pa}, *tagF*$^{Pa\text{-}GK}$, *tagF*$^{Pa\text{-}SDR}$,and *fha1*^{Pa} ORFs (without stop codon) were PCR-amplified with primers described in Supplementary Table S2 and respectively cloned into pGBKT7 or pGADT7 with appropriate enzyme sites to create the plasmids pGBKT7-TagF-PppA, pGBKT7-TagF, pGBKT7-TagF^{GK}, pGBKT7-TagF^{DF}, pGBKT7-TagF^{DW}, pGBKT7-TagF^{SDR}, pGBKT7-TagF^{FD}, pGBKT7-TagF^{Pa}, pGBKT7-TagF$^{Pa\text{-}GK}$, pGBKT7-TagF$^{Pa\text{-}SDR}$, pGBKT7-Fha1^{Pa}, pGADT7-Fha, pGADT7-TagF, pGADT7-TagF^{Pa}, and pGADT7-Fha1^{Pa}, respectively.

For the constructs used for bacterial two-hybrid, the *tagF*^{Pa} and *fha1*^{Pa} ORFs (without stop codon) were PCR-amplified with primers described in Supplementary Table S2 and respectively cloned into pKT25 (8) or pUT18C (8) with appropriate enzyme sites to create the plasmids pKT25-TagF^{Pa}, pKT25-Fha1^{Pa}, pUT18C-TagF^{Pa}, and pUT18C-Fha1^{Pa}, respectively.

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6

Table S1. Bacterial strains and plasmids

Strain /plasmid	Relevant characteristics	Source/ reference
A. tumefaciens		
C58	Wild type virulent strain containing nopaline-type Ti plasmid pTiC58	Eugene Nester
EML1060	*ppkA*(*atu4330*) in frame deletion mutant, C58Δ*ppkA*	(1)
EML1063	*tagF-pppA* (*atu4331*) in frame deletion mutant, C58Δ*tagF-pppA*	(1)
EML4307	*ppkA* and *tagF-pppA* in-frame deletion mutant, C58Δ*ppkA*Δ*tagF-pppA*	This study
EML1073	*tssL* (*atu4333*) in-frame deletion mutant, C58Δ*tssL*	(2)
EML3561	Deletion from *atu3639* to *atu3640*, *atu4350* to *atu4351*, and *atu4346* to *atu4347*, C58Δ*3TIs*	(3)
EML4515	*tagF-pppA* with G22A and K23A substitutions, C58*tagF*^{GK}*-pppA*	This study
EML4517	*tagF-pppA* with D44A and W46A substitutions, C58*tagF*^{DW}*-pppA*	This study
EML4519	*tagF-pppA* with S93A, D95A and R99A substitutions, C58*tagF*^{SDR}*-pppA*	This study
P. aeruginosa		
PAKΔ*retS*	In-frame deletion of *retS* (PA4856) in PAK	(4)
PAKΔ*retS*Δ*H1*	Deletion of *retS* and H1-T6SS cluster in PAK	(5)
PAKΔ*retS*Δ*tssB1*	In-frame deletion of *retS* and *tssB1* (PA0083) in PAK	(6)
E. coli		
DH10B	Host for DNA cloning	Invitrogen
DH5α	Host for DNA cloning	Laboratory collection
BL21 (DE3)	Host for overexpressing genes driven by the T7 promoter	(7)
S. cerevisiae		
AH109	Host for yeast two-hybrid analysis	Clontech
Plasmids		
pRL662	Gm^R, broad-host range vector derived from pBBR1MCS-2	(8)
pJQ200KS	Gm^R, suicide plasmid containing Gm^r and *sacB* gene for selection of double crossover	(9)
pET28a(+)	Km^R, *E. coli* overexpression vector to produce N or C-terminal His-tagged protein	Novagen
pTrc200	Sp^R , pVS1 origin *lacI*^{q}, *trc* promoter expression vector	(10)

pCR2.1	Ap^R, Km^R, TA cloning vector	Invitrogen
pGADT7	Ap^R, AD vector used in yeast two-hybrid assay	Clontech
pGBKT7	Km^R, DNA-BD vector used in yeast-two hybrid assay	Clontech
pKT25	Km^R, BTH vector for fusion of target proteins to *B. pertussis cya* gene T25 fragment; P_{lac}::$cya^{1–675}$ p15ori	(11)
pUT18C	Ap^R, BTH vector for fusion of target proteins to *B. pertussis cya* gene T18 fragment; P_{lac}::$cya^{675–1197}$ pUCori	(11)
$pTagF^{Pa}$	Gm^R, pRL662 expressing *P. aeruginosa* TagF driven by *lacZp*	This study
$pTagF^{Pa}$-Strep	Gm^R, pRL662 expressing *P. aeruginosa* TagF-Strep fusion protein driven by *lacZp*	This study
$pTagF^{Pa}$-^{GK}-Strep	Gm^R, pRL662 expressing *P. aeruginosa* TagF-Strep fusion protein with G8A and K9A substitutions driven by *lacZp*	This study
$pTagF^{Pa}$-^{SDR}-Strep	Gm^R, pRL662 expressing *P. aeruginosa* TagF-Strep fusion protein with S79A, D81A, and R85A substitutions driven by *lacZp*	This study
pTssL-His	Gm^R, pRL662 expressing TssL-His fusion protein driven by *lacZp*	(12)
pTrc-TagF-PppA	Sp^R, pTrc200 expressing TagF-PppA full-length protein	This study
pTrc-TagF	Sp^R, pTrc200 expressing TagF domain (1-230 a.a.)	This study
pTrc-TagF-Strep	Sp^R, pTrc200 expressing TagF-Strep fusion protein	This study
pTrc-$TagF^{GK}$-Strep	Sp^R, pTrc200 expressing TagF-Strep fusion protein with G22A and K23A substitutions	This study
pTrc-$TagF^{DF}$-Strep	Sp^R, pTrc200 expressing TagF-Strep fusion protein with D29A and F30A substitutions	This study
pTrc-$TagF^{DW}$-Strep	Sp^R, pTrc200 expressing TagF-Strep fusion protein with D44A and W46A substitutions	This study
pTrc-$TagF^{SDR}$-Strep	Sp^R, pTrc200 expressing TagF-Strep fusion protein with S93A, D95A and R99A substitutions	This study
pTrc-$TagF^{FD}$-Strep	Sp^R, pTrc200 expressing TagF-Strep fusion protein with F141A and D142A substitutions	This study
pTrc-PppA	Sp^R, pTrc200 expressing PppA domain (231-471 a.a.)	This study
pTrc-$Fha1^{Pa}$-HA	Sp^R, pTrc200 expressing *P. aeruginosa* Fha1-HA fusion protein	This study
pGBKT7-TagF-PppA	Km^R, DNA-BD vector expressing TagF-PppA	This study
pGBKT7-TagF	Km^R, DNA-BD vector expressing TagF domain (1-230 a.a.)	This study
pGBKT7-$TagF^{GK}$	Km^R, DNA-BD vector expressing TagF domain (1-230 a.a.) with G22A and K23A substitutions	This study

pGBKT7-TagF^{DF}	Km^{R}, DNA-BD vector expressing TagF domain (1-230 a.a.) with D29A and F30A substitutions	This study
pGBKT7-TagF^{DW}	Km^{R}, DNA-BD vector expressing TagF domain (1-230 a.a.) with D44A and W46A substitutions	This study
pGBKT7-TagF^{SDR}	Km^{R}, DNA-BD vector expressing TagF domain (1-230 a.a.) with S93A, D95A and R99A substitutions	This study
pGBKT7-TagF^{FD}	Km^{R}, DNA-BD vector expressing TagF domain (1-230 a.a.) with F141A and D142A substitutions	This study
pGBKT7-TagF^{Pa}	Km^{R}, DNA-BD vector expressing *P. aeruginosa* TagF	This study
pGBKT7-TagF$^{Pa\text{-}GK}$	Km^{R}, DNA-BD vector expressing *P. aeruginosa* TagF with G8A and K9A substitutions	This study
pGBKT7-TagF$^{Pa\text{-}SDR}$	Km^{R}, DNA-BD vector expressing *P. aeruginosa* TagF with S79A, D81A, and R85A substitutions	This study
pGBKT7-Fha1^{Pa}	Km^{R}, DNA-BD vector expressing *P. aeruginosa* Fha1	This study
pGBKT7-53	Km^{R}, DNA-BD vector expressing murine p53	Clontech
pGADT7-TagF	AP^{R}, AD vector expressing TagF domain (1-230 a.a.)	This study
pGADT7-Fha	AP^{R}, AD vector expressing Fha	This study
pGADT7-TagF^{Pa}	AP^{R}, AD vector expressing *P. aeruginosa* TagF	This study
pGADT7-Fha1^{Pa}	AP^{R}, AD vector expressing *P. aeruginosa* Fha1	This study
pGADT7-T	Ap^{R}, AD vector expressing SV40 large T-antigen	Clontech
pKT25-TagF^{Pa}	Km^{R}, fusion of *tagF^{Pa}* to *cya* gene T18 fragment in pKT25	This study
pKT25-Fha1^{Pa}	Km^{R}, fusion of *fha1^{Pa}* to *cya* gene T18 fragment in pKT25	This study
pUT18C-TagF^{Pa}	Ap^{R}, fusion of *tagF^{Pa}* to *cya* gene T25 fragment in pUT18C	This study
pUT18C-Fha1^{Pa}	Ap^{R}, fusion of *fha1^{Pa}* to *cya* gene T25 fragment in pUT18C	This study
pJQ200KS-Δ*ppkA*Δ*tagF-pppA*	Gm^{R}, used in generating *ppkA* and *tagF-pppA* double deletion mutant of *A. tumefaciens* C58	This study
pJQ200KS- *tagF^{GK}-pppA*	Gm^{R}, used in generating *tagF-pppA* with G22A and K23A substitutions of *A. tumefaciens* C58	This study
pJQ200KS- *tagF^{DW}-pppA*	Gm^{R}, used in generating *tagF-pppA* with D44A and W46A substitutions of *A. tumefaciens* C58	This study
pJQ200KS- *tagF^{SDR}-pppA*	Gm^{R}, used in generating *tagF-pppA* with S93A, D95A, and R99A substitutions of *A. tumefaciens* C58	This study
pET28a(+)-*tagF* 1-214	Km^{R}, pET28a(+) expressing N-terminal His-tagged TagF 1-214 aa protein	This study

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Table S2. Primer information

Primer	Plasmids	Sequence (5 '-3')[a]	Source / reference
TagF-PppA F-BamHI	pTrc-TagF	5'-CGGGATCCTTATGATGAAGGCAAGCACG-3'	(1)
TagF N1-230 R-XbaI		5'-GCTCTAGACTATGTCTTCTCCTGCACCGTTGC-3'	This study
TagF-PppA F-BamHI	pTrc-TagF-Strep	5'-CGGGATCCTTATGATGAAGGCAAGCACG-3'	(1)
TagF N1-230-Strep R-XbaI		5'-GCTCTAGACTACTTTTCGAACTGCGGGTGGCTCCATGTCTTCTCCTGCACCGTTGC-3'	This study
TagF-PppA F-BamHI	pTrc-TagF^{GK}-Strep	5'-CGGGATCCTTATGATGAAGGCAAGCACG-3'	(1)
TagF N1-230-Strep R-XbaI		5'-GCTCTAGACTACTTTTCGAACTGCGGGTGGCTCCATGTCTTCTCCTGCACCGTTGC-3'	This study
TagF GK-1		5'-CCATGGCTGGGGACC**GC**G**G**CGAAGAAACCGATGC-3'	This study
TagF GK-2		5'-GCATCGGTTTCTTCG**C**C**GC**GGTCCCCAGCCATGG-3'	This study
TagF-PppA F-BamHI	pTrc-TagF^{DF}-Strep	5'-CGGGATCCTTATGATGAAGGCAAGCACG-3'	(1)
TagF N1-230-Strep R-XbaI		5'-GCTCTAGACTACTTTTCGAACTGCGGGTGGCTCCATGTCTTCTCCTGCACCGTTGC-3'	This study
TagF DF-1		5'-GAGCCCATCGGAAATG**GC**A**G**CGCCATGGCTGGGGAC-3'	This study
TagF DF-2		5'-GTCCCCAGCCATGGCG**C**T**GC**CATTTCCGATGGGCTC-3'	This study
TagF-PppA F-BamHI	pTrc-TagF^{DW}-Strep	5'-CGGGATCCTTATGATGAAGGCAAGCACG-3'	(1)
TagF N1-230-Strep R-XbaI		5'-GCTCTAGACTACTTTTCGAACTGCGGGTGGCTCCATGTCTTCTCCTGCACCGTTGC-3'	This study
TagF DW-1		5'-CATGCCGGATC**GC**ATCGCGTCG**G**CGAAGGTAGCGATCA-3'	This study
TagF DW-2		5'-TGATCGCTACCTTCG**C**CGAC**GC**GATGCGATCCGGCATG-3'	This study
TagF-PppA F-BamHI	pTrc-TagF^{SDR}-Strep	5'-CGGGATCCTTATGATGAAGGCAAGCACG-3'	(1)
TagF N1-230-Strep R-XbaI		5'-GCTCTAGACTACTTTTCGAACTGCGGGTGGCTCCATGTCTTCTCCTGCACCGTTGC-3'	This study
TagF SDR-1		5'-GGATATTTA**GC**GCCCACCCG**GG**CGGCG**GC**CGGCACCAGCACG-3'	This study
TagF SDR-2		5'-CGTGCTGGTGCCG**GC**CGCCG**C**CCGGGTGGGC**GC**TAAATATCC-3'	This study
TagF-PppA F-BamHI	pTrc-TagF^{FD}-Strep	5'-CGGGATCCTTATGATGAAGGCAAGCACG-3'	(1)
TagF N1-230-Strep R-XbaI		5'-GCTCTAGACTACTTTTCGAACTGCGGGTGGCTCCATGTCTTCTCCTGCACCGTTGC-3'	This study
TagF FD-1		5'-CATTAAACCGGGACACG**G**CG**GC**ATCGCCGGTCATCGAT-3'	This study
TagF FD-2		5'-ATCGATGACCGGCGAT**GC**CG**C**CGTGTCCCGGTTTAATG-3'	This study
TagF^{Pa} F-XhoI	pTagF^{Pa}	5'-CCGCTCGAGCCAGCATCGCCTGCGAGCTGC-3'	This study
TagF^{Pa} R-XbaI		5'-GCTCTAGACGACCTGTAGTAGCTGACTGAG-3'	This study
TagF^{Pa} F-XhoI	pTagF^{Pa}-Strep	5'-CCGCTCGAGCCAGCATCGCCTGCGAGCTGC-3'	This study
TagF^{Pa} Strep R-XbaI		5'-GCTCTAGACTACTTTTCGAACTGCGGGTGGCTCCAACCGGGTATGCCGGGAAAGAGC-3'	This study
TagF^{Pa} F-XhoI	pTagF$^{Pa\text{-}GK}$-Strep	5'-CCGCTCGAGCCAGCATCGCCTGCGAGCTGC-3'	This study
TagF^{Pa} Strep R-XbaI		5'-GCTCTAGACTACTTTTCGAACTGCGGGTGGCTCCAACCGGGTATGCCGGGAAAGAGC-3'	This study
TagF^{Pa} GK-1		5'-CCGCGGCCGGCCAGC**GC**G**G**CGTAGAAACCGACGC-3'	This study

TagF^{Pa} GK-2		5'-GCGTCGGTTTCTACG**C**C**GC**GCTGGCCGGCCGCGG-3'	This study
TagF^{Pa} F-XhoI	pTagF^{Pa-SDR}-Strep	5'-CCGCTCGAGCCAGCATCGCCTGCGAGCTGC-3'	This study
TagF^{Pa} Strep R-XbaI		5'-GCTCTAGACTACTTTTCGAACTGCGGGTGGCTCCAACCGGGTATGCCGGGAAAGAGC-3'	This study
TagF^{Pa} SDR-1		5'-GGGAAATAG**GC**ACCGACCCGG**G**CGATG**GC**CGGCATCACCACT-3'	This study
TagF^{Pa} SDR-2		5'-AGTGGTGATGCCG**GC**CATCG**C**CCGGGTCGGT**GC**CTATTTCCC-3'	This study
PppA C231-471-BamHI	pTrc-PppA	5'-CGGGATCCATGAAACAGCAAATACCGCCCGTG-3'	This study
TagF-PppA R-XbaI		5'-GCTCTAGAGGATAGGCAGGCTCATCAAG-3'	(1)
TagF-PppA F-BamHI	pTrc-TagF-PppA	5'-CGGGATCCTTATGATGAAGGCAAGCACG-3'	(1)
TagF-PppA R-XbaI		5'-GCTCTAGAGGATAGGCAGGCTCATCAAG-3'	(1)
Fha1^{Pa} F-NcoI	pTrc-Fha1^{Pa}-HA	5'-CATGCCATGGCCTGGATGCGAACCGAAATCC-3'	This study
Fha1^{Pa} HA R-XbaI		5'-GCTCTAGATCAAGCGTAATCTGGAACATCGTATGGGTAGGAACGCCGTAGTCGAGCGCTG-3'	This study
TagF-PppA F	pGBKT7-TagF-PppA	5'-TGGCCGATCAGGCATCAAGG-3'	(2)
TagF-PppA R-BamHI		5'-CGGGATCCGCCTTGCTCACGCCGGTTTC-3'	This study
TagF-PppA F	1. pGBKT7-TagF	5'-TGGCCGATCAGGCATCAAGG-3'	(2)
TagF R-BamHI	2. pGBKT7-TagF^{GK} (pTrc-TagF^{GK}-Strep as template) 3. pGBKT7-TagF^{DF} (pTrc-TagF^{DF}-Strep as template) 4. pGBKT7-TagF^{DW} (pTrc-TagF^{DW}-Strep as template) 5. pGBKT7-TagF^{SDR} (pTrc-TagF^{SDR}-Strep as template) 6. pGBKT7-TagF^{FD} (pTrc-TagF^{FD}-Strep as template) 7. pGADT7-TagF	5'-CGGGATCCTGTCTTCTCCTGCACCGTTGC-3'	This study
Fha F-NdeI	pGADT7-Fha	5'-GAACATATGAAGCTTGCACTCAAGAACAC-3'	(2)
Fha R-BamHI		5'-CGGGATCCTGTCTCATCGTGGTTGTTTACC-3'	This study
TagF^{Pa} F	1. pGBKT7-TagF^{Pa}	5'-TGTTGAACAGCGTCGGTTTCTACG-3'	This study
TagF^{Pa} R-BamHI	2. pGBKT7-TagF^{Pa-GK} (pTrc-TagF^{Pa-GK}-Strep as template) 3. pGBKT7-TagF^{Pa-SDR} (pTrc-TagF^{Pa-SDR}-Strep as template) 4. pGADT7-TagF^{Pa}	5'-CGGGATCCACCGGGTATGCCGGGAAAGAGC-3'	This study
Fha1^{Pa} F	1. pGBKT7-Fha1^{Pa}	5'-TGCCGCTGCGATTGACCATCAC-3'	This study
Fha1^{Pa} R-BamHI	2. pGADT7-Fha1^{Pa}	5'-CGGGATCCGGAACGCCGTAGTCGAGCGCTG-3'	This study
TagF^{Pa} F-XbaI	1. pKT25-TagF^{Pa} 2. pUT18C-TagF^{Pa}	5'-GCGCGTCTAGAGTTGAACAGCGTCGGTTTCTACG-3'	This study
TagF^{Pa} R-EcoRI		5'-GCGCGGAATTCTTAACCGGGTATGCCGGGA-3'	This study
Fha1^{Pa} F-XbaI	1. pKT25-Fha1^{Pa} 2. pUT18C-Fha1^{Pa}	5'-GTTAGTCTAGAGATGCCGCTGCGATTGACCAT-3'	This study
Fha1^{Pa} R-BamHI		5'-AATACGGATCCTCAGGAACGCCGTAGTCGAG-3'	This study
PpkA 2F-BamHI	pJQ200KS-Δ*ppkA*Δ*tagF-pppA*	5'-CGGGATCCCTGTAGCGCCGGCGTCAGTTG-3'	(2)
PpkA 2R-XmaI		5'-TCCCCCCGGGCCCGTCAGGAGCGTGTACTTG-3'	(2)
TagF-PppA 1F-XbaI		5'-GCTCTAGAGCCCAGTTCGAAAATGCCGAC-3'	(2)
TagF-PppA 1R-BamHI		5'-CGGGATCCATCGGCCATCAGTTGCGATTG-3'	(2)
TagF-PppA 1F-XbaI	pJQ200KS-*tagF*^{GK}*-pppA*	5'-GCTCTAGAGCCCAGTTCGAAAATGCCGAC-3'	(2)
TagF-PppA 2R-XmaI		5'-TCCCCCCGGGCGAAGGATCGAGATCACCTGC-3'	(2)
TagF GK-1		5'-CCATGGCTGGGGACC**GC**G**G**CGAAGAAACCGATGC-3'	This study

TagF GK-2		5'-GCATCGGTTTCTTCG**CCGC**GGTCCCCAGCCATGG-3'	This study
TagF-PppA 1R		5'-GATCGATGTGCCACCAGAGG-3'	This study
TagF-PppA 1F-XbaI	pJQ200KS-*tagF*^{DW}-*pppA*	5'-GCTCTAGAGCCCAGTTCGAAAATGCCGAC-3'	(2)
TagF-PppA 2R-XmaI		5'-TCCCCCCGGGCGAAGGATCGAGATCACCTGC-3'	(2)
TagF DW-1		5'-GAGCCCATCGGAAATG**GC**A**G**CGCCATGGCTGGGGAC-3'	This study
TagF DW-2		5'-GTCCCCAGCCATGGCG**C**T**GC**CATTTCCGATGGGCTC-3'	This study
TagF-PppA 1R		5'-GATCGATGTGCCACCAGAGG-3'	This study
TagF-PppA 1F-XbaI	pJQ200KS-*tagF*^{SDR}-*pppA*	5'-GCTCTAGAGCCCAGTTCGAAAATGCCGAC-3'	(2)
TagF-PppA 2R-XmaI		5'-TCCCCCCGGGCGAAGGATCGAGATCACCTGC-3'	(2)
TagF SDR-1		5'-GGATATTTA**GC**GCCCACCCG**GG**CGGCG**GC**CGGCACCAGCACG-3'	This study
TagF SDR-2		5'-CGTGCTGGTGCCG**GC**CGCCG**CC**CGGGTGGGC**GC**TAAATATCC-3'	This study
TagF-PppA 1R		5'-GATCGATGTGCCACCAGAGG-3'	This study
TagF 1-214 F	pET28a(+)-*tagF* 1-214	5'-CAGGCAAAGGCGACGAGTAACAAAGCCCGAAAG-3'	This study
TagF 1-214 R		5'-CTTTCGGGCTTTGTTACTCGTCGCCTTTGCCTG-3'	This study

a: Restriction enzyme sites are underlined, and mutated sequences are indicated by bold type.

1. Lin, J. S., Wu, H. H., Hsu, P. H., Ma, L. S., Pang, Y. Y., Tsai, M. D., and Lai, E. M. (2014) Fha interaction with phosphothreonine of TssL activates type VI secretion in Agrobacterium tumefaciens. *PLoS Pathog* **10**, e1003991
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