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農桿菌第六型分泌系統效應子裝載機制的研究

Exploring the Effector Loading Mechanisms of a Type VI Secretion System in *Agrobacterium tumefaciens*

連允薇

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ABSTRACT

Type VI secretion system (T6SS) is a molecular machinery widespread in Gram-negative bacteria including the causal agent of plant crown gall disease, *Agrobacterium tumefaciens*. A T6SS machine is composed of a membrane complex [Tss(J)LM], a baseplate [Tss(A)EFGK], and a contractile tube consisting an Hcp-VgrG inner tube wrapped by an outer TssBC sheath. This nanomachine has been deployed to deliver diverse effector proteins into eukaryotic host cells or bacterial competitors to increase the fitness of T6SS-possessing bacteria. *A. tumefaciens* strain C58 encodes one main T6SS gene cluster comprising *imp* operon and *hcp* operon as well as one orphan *vgrG2* operon encoded elsewhere. Three toxin-immunity pairs (Tae-Tai, Tde1-Tdi1 and Tde2-Tdi2) were produced. Among them, Tde1 and Tde2 are DNase toxins and Tae is a putative peptidoglycan amidase. These Tde DNase effectors are delivered by a VgrG-specific manner, in which Tde1 and Tde2 are cargo effectors of VgrG1 and VgrG2, respectively.

Chaperone/adaptor proteins are also involved in effector loading to VgrG because Tap-1 interacts with Tde1 for binding to VgrG1 tip and Atu3641 is required for VgrG2-mediated Tde2 delivery. Nevertheless, how this effector-VgrG complex is loaded onto a T6SS machine for secretion still remains unknown. Secretion of Hcp

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tube and VgrG spike is regarded as a hallmark of T6SS firing. Though it is widely believed that cargo effectors (or non-VgrG/Hcp effectors) are not a component of a Type IV secretion apparatus, we found that deletion of *tde1-tdi1* or *tde2-tdi2* diminishes VgrG1 and VgrG2 secretion, respectively. Furthermore, VgrG1 secretion is abolished in Δ*tap-1* whereas VgrG2 is no longer secreted in Δ*atu3641*. These findings suggest that Tde effector loaded onto its cognate VgrG is required for assembly of a functional T6SS for secretion. Next, I determined how VgrG-effector complex is loaded onto membrane-associated T6SS subcomplex for T6SS firing. To test whether VgrG and VgrG-Tde complex interact with baseplate components, protein-protein interaction studies were carried out by co-purification in *Escherichia coli* and in *A. tumefaciens*. *E. coli* co-purification assay results showed that among the baseplate proteins, VgrG interacts with TssA, TssF, TssG and TssK but not TssE. Higher levels of VgrG1-TssK interactions were detected when co-expression of Tap-1, Tde1, Tdi1 and PAAR in *E. coli*, suggesting that Tde1 loaded onto VgrG1 enhances VgrG1 interaction to TssK. In *A. tumefaciens*, TssA and TssK but not TssE are co-purified with His-tagged VgrG1 or VgrG2 proteins. On the other hand, His-tagged TssK is able to co-purify TssA, TssC, TssM and VgrG1-Tde1 complex in wild type *A. tumefaciens*. Interestingly, TssK no longer interacts with TssM in the absence

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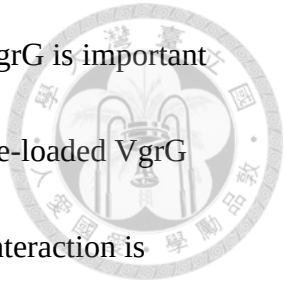

of any Tde effectors, suggesting that Tde loading onto its cognate VgrG is important for TssK-TssM interaction. Taken together, we suggest that only Tde-loaded VgrG can bind strongly to baseplate component TssK and such tripartite interaction is required for TssK recruitment to TssM-TssL membrane complex for initiation of T6SS assembly and firing. We propose that this is a strategy utilized by *A. tumefaciens* and likely also other T6SS-possessing bacteria to save energy when VgrG cargo effector is not loaded.

Other than VgrG cargo effector Tde, Tae that is likely to be the Hcp cargo effector was found to regulate T6SS through a different way. In acidic minimal medium, deletion of *tae* locus results in Hcp secretion restoration in *tde-tdi* double deletion mutant. It is possible that beside being a bacterial toxin, Tae may also function as a protein controlling the length of Hcp tube and TssBC sheath or preventing Hcp tube from sliding out during contraction, similar to tape measure protein or capping protein of bacteriophage.

Keywords: *Agrobacterium tumefaciens*, type VI secretion system, VgrG, TssK, Tde, effector loading, protein-protein interaction

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中文摘要

第六型分泌系統存在於許多革蘭氏陰性菌中，包括造成植物癌腫病的農桿菌。第六型分泌系統由四個構造組成，膜蛋白質複合體 TssLM 或 TssJLM、底座 Tss(A)EFGK、管狀構造 Hcp 與 VgrG 與可伸縮的外鞘 TssBC。第六型分泌系統可以藉由分泌效應子到宿主細胞或是細菌競爭者中來提供病原性或提高適應性。農桿菌品系 C58 具有一個第六型分泌系統基因簇，包括 *imp* 操縱組與 *hcp* 操縱組，與一個位於染色體其他地方的孤兒 *vgrG2* 操縱組。農桿菌品系 C58 總共能產生三個效應子免疫蛋白質對，Tae-Tai、Tde1-Tdi1 和 Tde2-Tdi2。Tae 被預測為一個肽聚醣醯胺酶，而 Tde1 和 Tde2 則為 DNA 分解酶。先前的研究顯示 Tde1 和 Tde2 會專一的由 VgrG1 和 VgrG2 運送，且 Tde1 需要 Tap-1 伴侶蛋白質，而 Tde2 需要 Atu3641 等伴侶蛋白質才能夠被分泌並維持其穩定性。然而，我們仍然不知道 VgrG-Tde 複合體如何被裝載至第六型分泌系統。VgrG 和 Hcp 的分泌是第六型分泌系統功能性的一個重要指標。目前普遍認為非 VgrG 和 Hcp 的第六型分泌系統效應子並不是第六型分泌系統的一個零件，然而我們發現，刪除 *tde1-tdi1* 或 *tde2-tdi2* 將分別導致 VgrG1 或 VgrG2 的分泌消失。當 *tde1-tdi1* 和 *tde2-tdi2* 這兩個效應子免疫蛋白質基因對都被刪除時，Hcp 的分泌將大量下降，而 VgrG 的分泌則完全無法被偵測。且 *tap-1* 突變株只有 VgrG2 的分泌，而 *atu3641* 突變株只能分泌 VgrG1。這些發現讓我們假設第六型分泌

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系統效應子的裝載對 VgrG 與其他第六型分泌系統底座蛋白質的交互作用與組裝是重要的。接著，為了瞭解 VgrG 與其他底座蛋白質的交互作用，我們在大腸桿菌或是農桿菌中進行組胺酸標定(Histidine tag)的 VgrG1 的共純化實驗。在大腸桿菌中，我們發現 VgrG1 可以和 TssA、TssF、TssG 及 TssK 有交互作用，而與 TssE 沒有。而當 Tap-1、Tde1 和 VgrG1-His 與 TssK 一起表現在大腸桿菌中時，和沒有表現 Tde1 與 Tap-1 比起來，有更多的 TssK 被 VgrG1-His 共純化。在農桿菌中，組胺酸標定的 VgrG1 或 VgrG2 能夠共沉澱 TssA 與 TssK，但無法共沉澱 TssE。且組胺酸標定的 TssK 能夠共沉澱 TssA、TssC、TssM 與 VgrG1-Tde1 複合體。有趣的是，當兩個 Tde 都不存在時， TssK 不再與 TssM 有交互作用。綜合以上的結果，我們推測只有有裝載 Tde 的 VgrG 能夠有效的和 TssK 進行交互作用，且 TssK 與 TssM 之間的交互作用需要這三個蛋白質形成的複合體；而第六型分泌系統只有在 VgrG 上有裝載 Tde 效應子時才能夠有效的發射。我們認為這可能是農桿菌或其他具有第六型分泌系統的細菌在細胞內沒有效應子蛋白質時所做出的節省能量的策略。

除了藉由 VgrG 來運送的 Tde，我們發現藉由 Hcp 運送的 Tae 可能具有與 Tde 不同的調控第六型分泌系統的方式。我們發現，刪除 *tae* 的基因將會導致 *tde-tdi* 雙突變株的 Hcp 分泌量上升。這有可能是因為 Tae 可以扮演類似噬菌體中的

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tape measure protein 或是 capping protein 的功能。這兩個蛋白質能夠控制噬菌體的鞘的長度或是防止管狀構造在伸縮的時候滑出。

關鍵字：農桿菌、第六型分泌系統、VgrG、TssK、Tde、效應子裝載、蛋白質交互作用

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INTRODUCTION

1. Bacterial protein secretion systems

1.1 General secretion pathways

To thrive in its ecological niche, a bacterium may secrete small molecules such as antibiotics or macro molecules such as proteins or nucleic acids out from its cell to the surrounding milieu for its survival and competitive growth. Bacteria have therefore evolved to assemble several "machines" for export or secretion, among them the namely protein secretion systems are the major players. Protein secretion systems can be divided into two types; the general secretion pathways, including Sec pathway and twin-arginine (Tat) pathway, and the specialized secretion systems, including type I to type IX secretion systems (Natale et al., 2008; Green and Mecsas, 2016; Maffei et al., 2017). The general secretion pathway, Sec or Tat, are universally distributed among bacteria, archaea and eukaryotes. Sec pathway is responsible for translocating unfolded protein substrate to the periplasm or inner membrane of the bacteria, while Tat pathway transfers fully folded proteins to the periplasm (Natale et al., 2008; Green and Mecsas, 2016). These general secretion systems are featured by their N-terminal signal peptides that harbors a polar "n region" (short positively sequence) located at the N-terminus, a

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central region “h region” composed of 7-15 hydrophobic residues, and a “c region” containing an AXA cleavage site motif. Upon translocation across inner membrane into the periplasm, the signal peptide is removed at this cleavage site (Preston et al., 2005). The signal peptide of Tat pathway shares similar features to Sec pathway but contains a conserved pattern of two almost invariant arginines at the interface of the n- and h-region, therefore named as “twin-arginine pathway” (Müller, 2005).

Some of the specialized secretion systems require Sec or Tat pathway to accomplish protein translocation; these secretion systems are called two-step secretion systems, including type II, type V and type IX secretion systems; others that do not rely on Sec or Tat for translocation and likely export directly through the specialized protein secretion channel are called one-step secretion systems (Green and Mecsas, 2016; Maffei et al., 2017).

1.2 Type I secretion system

Type I secretion system (T1SS) is widely present in many pathogenic Gram-negative bacteria, such as *Vibrio cholerae*, uropathogenic *Escherichia coli,* and *Bordetella pertussis* (Thomas et al., 2014). A T1SS machine is composed of three proteins, an ATP-binding cassette transporter, a membrane fusion protein, and an outer membrane pore

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channel protein. Its substrates are characterized by their non-cleaved C-terminal secretion signal (Gray et al., 1986; Gray et al., 1989).

1.3 Type II secretion system

Type II secretion system (T2SS) is a two-step secretion system, in which Sec or Tat pathway is required for the substrate translocation across inner membrane into periplasm before secreted out from the cell by T2SS (Douzi et al., 2012). It is structurally and evolutionally related to type IV pilus system, archaeal flagella and the transformation system in Gram-positive bacteria (Hobbs and Mattick, 1993; Korotkov et al., 2012). A T2SS machinery can be divided into several components, an outer-membrane complex, a pseudopilus, an inner-membrane platform, and a secretion ATPase (Korotkov et al., 2012). The T2SS substrates were proposed to firstly interact with outer-membrane protein GspD, inner-membrane protein GspC and/or the tip of the pseudopilus, and they will be pushed out from the cell by growing pseudopilus (Hobbs and Mattick, 1993; Shevchik et al., 1997). T2SS is widespread among bacterial pathogens causing diseases in plants and animals including human. Substrates delivered by T2SS have diverse biochemical activities, including a wild range of hydrolases able to hydrolyze proteins, polysaccharides or lipids (Jha et al., 2005). These hydrolase

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effectors contribute virulence to lots of plant pathogen, including *Dickeya dadantii*, *Ralstonia solanacearum* and *Xanthomonas campestris*.

1.4 Type III secretion system

A type III secretion system (T3SS) injectisome shares common components with flagella (Pallen et al., 2005; Galán et al., 2014; Diepold and Armitage, 2015). From the phylogenic analysis, a T3SS may be evolved from a flagellum machine that lost its motility function (Galán et al., 2014). A T3SS machine can be briefly divided into three parts, a basal body anchored on the membrane, a needle-like structure and a translocator binding with host cell surface (Cornelis, 2002; Büttner, 2012). Translocator is a unique structure which is able to deliver effector proteins into the target cells rather than the intracellular milieu (Cornelis, 2002; Büttner, 2012). Biological function of T3SS is often associated with virulence or symbiosis toward its host. There is a distinct feature that T3SS has a chaperone-mediated substrate switching event during its firing action which optimizes the timing of the effector translocation (Büttner, 2012).

1.5 Type IV secretion system

Type IV secretion system (T4SS) is a machine with diverse functions, including conjugation between bacteria, DNA uptake and release, and effector delivery into host

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cells (Fronzes et al., 2009; Zechner et al., 2012; Christie et al., 2014). A T4SS machine is composed of three major compartments, cell surface pili, a transport channel spanning two membranes, and a type IV coupling protein which recognizes the T4SS substrate at the cytoplasmic site of the T4SS machine (Zechner et al., 2012). Importantly, T4SS is used by *A. tumefaciens* to transport the T-DNA complex and effectors thus required for *Agrobacterium* virulence (Zechner et al., 2012; Lacroix and Citovsky, 2013).

1.6 Type V secretion system

Type V secretion system (T5SS), also known as autotransporter, is a two-step secretion apparatus requiring Sec pathway to deliver substrates (Leyton et al., 2012; van Ulsen et al., 2014). Substrates of T5SS is first transported to periplasm via Sec pathway, and then the beta-domain on the C-terminus of the substrate is inserted into the outer membrane. In some cases, the outer membrane Bam complex is required for translocation of the passenger domain of the substrate onto the cell surface. T5SS is known to mediate contact-dependent growth inhibition (CDI) in Gram-negative bacteria, which is a mechanism involved in competition at intra-species levels via translocation of T5SS substrates, such as RNase, DNase or pore-forming effectors (Jacob-Dubuisson et al., 2013; Ruhe et al., 2013).

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1.7 Other secretion systems

Type VI secretion system (T6SS) is a nanoweapon used to deliver diverse effector proteins into host cells to confer virulence, into bacterial competitors to increase the fitness, or into the surrounding environment to obtain some metal ions (Russell et al., 2014; Cianfanelli et al., 2016b; Lien and Lai, 2017). As a focus of this thesis, more comprehensive literature reviews on T6SS are described in details in the following section.

Unlike the secretion systems we introduced above that are only or mainly present in Gram(-) bacteria, type VII secretion system (T7SS) is possessed by Gram-positive bacteria, including *Mycobacterium*, *Staphylococcus* and *Bacillus*, etc. It is involved in virulence, interbacterial competition, and metal ion acquisition (Unnikrishnan et al., 2017). Type VIII secretion system is specific to the biogenesis of curli (Chapman et al., 2002). Type IX secretion (T9SS) is another two-step secretion system. It is only found in the phylum Bacteroidetes (McBride and Zhu, 2013). T9SS is associated with the pathogenicity of *Porphyromonas gngivalis*, a causal agent of periodontitis, and the gliding motility of other bacteria species in Bacteroidetes phylum (Sato et al., 2010).

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2.Type VI secretion system

2.1 Overview of T6SS

T6SS is encoded in about 25% of bacterial genomes sequenced to date (Boyer et al., 2009). Before the identification of T6SS gene cluster and the determination of T6SS biological function, Hcp secretion, regarded as a hallmark of functional T6SS, was first found in *V. cholerae* back to 1996 (Williams et al., 1996). Due to the feature that Hcp is secreted without cleavage, it was proposed that Hcp is secreted through a novel secretion pathway. Later on, a gene cluster containing 14 genes in *Rhizobium leguminosarum* was found to associate with the formation of functional nodules therefore named impaired in nitrogen fixation (*imp*) locus (Bladergroen et al., 2003), which is indeed a T6SS gene cluster. On the other hand, EvpC, homolog of Hcp in a fish pathogen *Edwardsiella tarda*, was also found to be secreted in a proteomics profile (Rao et al., 2004). In 2006, T6SS was documented to be a newly identified secretion system in *P. aeruginosa* and *V. cholerae* associated with the virulence toward eukaryotic host (Mougous et al., 2006; Pukatzki et al., 2006).

2.2 T6SS machine assembly

A T6SS gene cluster typically encodes about 13-14 conserved core genes for

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assembly of T6SS (*tss* representing type VI secretion), few to several genes for diverse effectors, and some accessory genes (*tag* representing type VI associated gene) encoding proteins associated with regulation or effector delivery (Records, 2011; Basler, 2015; Cianfanelli et al., 2016b). A T6SS machine can be divided into four parts, a Tss(J)LM membrane complex, a Tss(A)EFGK baseplate, a TssBC sheath and Hcp tube capped with VgrG spike and PAAR tip (Cianfanelli et al., 2016b). Current T6SS assembly model suggests that membrane complex likely forms first, which serves as a docking site for baseplate recruitment onto membrane followed by polymerization of Hcp-VgrG-PAAR puncturing device wrapped around by TssBC sheath (Basler et al., 2012; Brunet et al., 2014; Brunet et al., 2015; Durand et al., 2015; Zoued et al., 2017). Based on the experiments done in *V. cholerae*, once the machine fires for secretion of puncturing device and associated effectors, ClpV ATPase will bind to contracted TssBC sheath for disassembly and recycling of the machine components (Bonemann et al., 2009; Basler and Mekalanos, 2012).

T6SS and bacteriophage are evolutionally and structurally conserved (Pukatzki et al., 2007; Leiman et al., 2009; Bock et al., 2017). Several T6SS machine components are homologous or structurally similar to bacteriophage proteins (Table 1), such as VgrG

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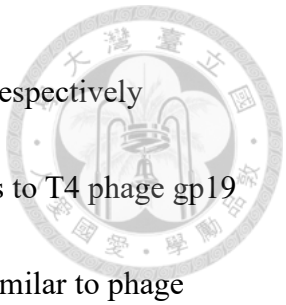

spike and PAAR tip are homologs of T4 phage gp27/gp5 and gp5.4, respectively (Pukatzki et al., 2007; Shneider et al., 2013); Hcp tube is homologous to T4 phage gp19 (Leiman et al., 2009); and TssBC forms a sheath that is structurally similar to phage gp18 (Leiman et al., 2009; Lossi et al., 2013; Kudryashev et al., 2015). For baseplate proteins, the situation is more complicated. *P. aeruginosa* TssA1 dodecamer structurally resembles T4 phage gp6 ring while enteroaggregarive *E. coli* (EAEC) TssA protein lacks the domain of phage protein (Planamente et al., 2016; Zoued et al., 2016; Zoued et al., 2017). Both of them contain an ImpA _N domain on N-terminal, while distinct domains were identified in their C-terminal region. A T4 phage gp6 domain was found in the C-terminus of *P. aeruginosa* TssA1, and a T6SS_VasJ domain was found in EAEC TssA C-terminus. As a result, the function of these TssA proteins are different. *P. aeruginosa* TssA1 interacts with sheath and other baseplate proteins and the transmission electron microscopy image indicates TssA1 is located at one end of the TssBC sheath. Combining with the structure information that it is homologous to T4 baseplate protein gp6, TssA1 was proposed to function as a connector belong to baseplate proteins between sheath-tube and baseplate-membrane complex. On the other hand, by visualizing super folding green fluorescence protein (sfGFP) tagged TssA, EAEC TssA protein was shown to be located at the distal end of TssBC sheath similar to

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the function of capping protein gp15 in T4 phage but not a baseplate protein (Zoued et al., 2016; Taylor et al., 2018). Furthermore, EAEC TssA is able to interact with some baseplate proteins, Hcp tube and sheath proteins, as well as form a complex with TssJLM subcomplex (Table 2). Therefore, EAEC TssA is proposed to facilitate the polymerization of Hcp tube and TssBC sheath at the distal end of sheath-tube structure. TssE and TssF are homologs of T4 phage gp25 and gp6 baseplate protein, respectively (Lossi et al., 2011; Brunet et al., 2015; Taylor et al., 2016). TssG is homologous to T4 phage gp53 in EAEC, gp53/gp7 in *V. cholerae* and gp7 in uropathogenic *E. coli* (Brunet et al., 2015; Taylor et al., 2016; Nazarov et al., 2017). By bacterial two hybrid (B2H) and co-IP using recombinant proteins expressed in *E. coli*, EAEC TssE was shown to interact with TssF and TssG (Brunet et al., 2015). These three proteins were co-immunoprecipitated with VgrG. Because TssFG complex has been shown to interact with TssK by B2H, the authors suggested that TssEFGK-VgrG form a baseplate in EAEC (Brunet et al., 2015). TssK is a trimeric protein, in which the EAEC TssK is structurally similar to siphophage receptor binding protein while *V. cholerae* and *P. aeruginosa* TssK proteins are homologous to gp10 and gp8 of T4 phage (Zoued et al., 2013; Planamente et al., 2016; Nazarov et al., 2017; Nguyen et al., 2017). In EAEC, TssK was proposed to connect the baseplate to the membrane complex and it is

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proposed to be the first baseplate protein recruited to membrane complex during T6SS machine assembly (Zoued et al., 2013; Brunet et al., 2015; Nguyen et al., 2017). TssFGK complex was identified by *in vivo* pull-down assay in *Serratia marcescens*, suggesting TssK is a member of baseplate components (English et al., 2014). Interestingly, the cryo-electron microscope (EM) image of extended sheath and baseplate of *V. cholerae* T6SS indicates that VgrG is surrounded by six TssK trimers, and the space between TssK and VgrG is proposed to accommodate VgrG cargo effectors (Nazarov et al., 2017). According to the protein-protein interaction data, cryo-EM reconstruction and the structure of a T4 phage baseplate, a T6SS baseplate may be assembled by a ring-like six-folded Tss(A)EFG inner baseplate with six TssK trimers connected to inner baseplate via interaction with TssFG. VgrG is located in the center of inner baseplate with its C-terminus toward TssK and membrane complex whereas VgrG cargo effectors are located at the space between VgrG and TssK trimer (English et al., 2014; Brunet et al., 2015; Planamente et al., 2016; Taylor et al., 2016; Nazarov et al., 2017; Nguyen et al., 2017b).

2.3 T6SS effector translocation

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T6SS effectors are transferred by T6SS via non-covalent interaction with VgrG, Hcp or PAAR proteins or being a C-terminus extension of VgrG, Hcp or PAAR (Cianfanelli et al., 2016b; Lien and Lai, 2017). Effectors that interact with T6SS VgrG or PAAR may locate at the space between VgrG-PAAR spike-tip and the surrounding TssK proteins (Nazarov et al., 2017). This space can accommodate one PAAR domain-containing effector up to ~450 kDa or three ~150 kDa VgrG cargo effectors in *V. cholerae* and may differ from bacterial species. Some of these effectors interact with VgrG directly, such as Tle1 in EAEC (Flaugnatti et al., 2016), which binds with VgrG C-terminus transthyretin (TTR)-like domain. Others may require an adaptor/chaperone protein for translocation and exhibit the antibacterial activity of the effectors, including Tap-1, Eag and DUF2169-containing protein (Alcoforado Diniz and Coulthurst, 2015; Liang et al., 2015; Unterweger et al., 2015; Whitney et al., 2015; Bondage et al., 2016; Cianfanelli et al., 2016a; Unterweger et al., 2016). In *V. cholerae* and *A. tumefaciens*, Tap-1, a DUF4123-containing protein is required for the interaction between cognate effector (such as TseL and Tde1) and VgrG (Liang et al., 2015; Bondage et al., 2016) and shown to stabilize the cognate effector as a chaperone protein (Ma et al., 2014; Liang et al., 2015). Recently, Tap-1 is also shown to facilitate the interaction between effector and PAAR (Burkinshaw et al., 2018). Eag is another T6SS adaptor/chaperone, and it is a

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DUF1789-containing protein shown to facilitate the interaction between VgrG with *P. aeruginosa* Tse6 and *S. marcescens* Rhs1 and Rhs2 effectors (Whitney et al., 2014; Whitney et al., 2015; Cianfanelli et al., 2016a; Unterweger et al., 2016). Although direct evidence has not been reported for DUF2169-containing protein interacting with VgrG or T6SS effectors, the VgrG2-mediated Tde2 antibacterial activity of *A. tumefaciens* is lost when the upstream DUF2169-containing protein (Atu3641) is absent (Bondage et al., 2016). Another type of effector-VgrG interaction is through the PAAR or PAAR-like, such as DUF4150 or DUF4280, domain in the N-terminus of the effector (Shneider et al., 2013; Bondage et al., 2016; Ma et al., 2016; Rigard et al., 2016). In addition to VgrG, T6SS effectors may be translocated by binding with Hcp in the lumen of Hcp tube (Silverman et al., 2013; Whitney et al., 2014; Cianfanelli et al., 2016a). It was shown in *P. aeruginosa* that Tse2 is localized inside Hcp tube, and Hcp confers a chaperone activity toward these Hcp-interacting effectors (Silverman et al., 2013). Similar phenomenon was also found in *S. marcescens*, in which Hcp1 affects the stability of effectors Ssp2 and Ssp4 (Cianfanelli et al., 2016a). To reside in the lumen of Hcp tube, these Hcp-interacting effectors are usually smaller proteins as compared with VgrG-interacting effectors.

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Other than interaction with T6SS machine components VgrG and Hcp, some VgrG or Hcp protein also carry effector function; termed as "specialized" VgrG or Hcp (Cianfanelli et al., 2016b). These specialized VgrG and Hcp possess effector domains at their C-terminus, which means they have dual functions as T6SS structure protein and T6SS effector. Specialized VgrG proteins were found in β- and γ-Proteobacteria including VgrG1 in *Aeromonas hydrophila*, VgrG-1 and VgrG-3 in *V. cholerae* and VgrG2b in *P. aeruginosa* (Pukatzki et al., 2006; Pukatzki et al., 2007; Ma and Mekalanos, 2010; Suarez et al., 2010; Brooks et al., 2013; Sana et al., 2015). On the other hand, although the idea that Hcp C-terminus may contain effector domain was proposed in 2009 (Boyer et al., 2009), such function was not proven until recently in Shiga toxin-producing *E. coli* that Hcp-ET1 has an DNase activity (Ma et al., 2017).

3. *Agrobacterium* biology

A. tumefaciens is a Gram-negative, rod shape (0.6–1.0 × 1.5–3.0 μm), aerobic bacterium with 1-4 peritrichous flagella, which belongs to Alphaproteobacteria (John M. Young, 2015). Type strain C58 contains one circular chromosome, one linear chromosome, one pAtC58 megaplasmid, and one pTiC58 megaplasmid (Allardet-

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Servent et al., 1993; Goodner et al., 2001; Wood et al., 2001).

A. tumefaciens was first identified as a causal agent of plant crown gall in 1907 (Smith and Townsend, 1907), and named as *Bacterium tumefaciens* at that time. It was surprising that once the pathogen infects a plant, agrobacteria are no longer required for tumor generation (White and Braun, 1941). Therefore, a term "tumor inducing principle (TIP)" was created to indicate that the tumor is caused by something produced by the bacteria but not the bacteria itself. The tumor cells isolated from "secondary tumor", tumor tissue with absence of agrobacteria, were further found to be able to grow on a medium without plant hormones, auxin and cytokinin, which are necessary for normal plant cells to grow on a medium (Braun, 1958). In 1960s, the virulence factor of *A. tumefaciens* was found to be transferred between virulent strains and avirulent strains (Kerr, 1969). In 1970s, a megaplasmid found to be exclusively present in virulent *A. tumefaciens* strain is isolated (Zaenen et al., 1974; Watson et al., 1975). This plasmid was further named as tumor inducing, or Ti plasmid. A small portion of the plasmid was found to be present in the tumor tissue, suggesting that TIP is indeed this fragment of DNA (Chilton et al., 1977). This DNA fragment was named as T-DNA for transferred DNA. Soon after the identification of T-DNA, it is further shown that T-DNA is

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transcribed in tumor (Drummond et al., 1977).

In addition, several types of opine were found to be produced in tumor, and the type of opine is consistent with the *A. tumefaciens* strain capable of acquiring this opine (Lippincott et al., 1972). Later, a genome-wide Tn5 transposon mutagenesis analysis in *A. tumefaciens* strain Ach5 was conducted (Garfinkel and Nester, 1980). It was found that insertion in the T-DNA region may result in defect of opine production or altered tumorigenesis but did not completely lost virulence. Insertion mutants with affected virulence were mapped to three major regions, two on Ti plasmid (T-DNA region and *vir* genes), one on circular chromosome, named as chromosomal virulence (*chv)* genes. DNA region responsible for triggering auxin or cytokinin synthesis in plant was mapped onto T-DNA by mutagenesis (Garfinkel et al., 1981; Ooms et al., 1981). At the same period of time, T-DNA was shown be inserted into the plant genome but not maintained as a plasmid form in tumor cells (Thomashow et al., 1980). The fact that *vir* gene expression is only observed *in planta* suggests that plant may play a role to induce *vir* gene transcription; and plant exudates, including phenolic compounds acetosyringone (AS), were found to induce *vir* genes expression (Stachel et al., 1985; Stachel et al., 1986a). VirA/VirG two-component is responsible for sensing AS (Lee et al., 1995).

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Other than phenolic compounds, several monosaccharides were also found to enhance *vir* gene expression via sugar binding protein ChvE. ChvE-monosaccharide complex interacts with VirA sensor kinase and results in activation of the transcription of *vir* genes (Ankenbauer and Nester, 1990; Cangelosi et al., 1990; Shimoda et al., 1990). Another environment signal associated with *vir* gene expression is pH. *virG* expression is also induced by acidic signal regulated by ChvG/ChvI two-component system (Charles and Nester, 1993; Mantis and Winans, 1993). Interestingly, ChvG/ChvI is not only used to induce *vir* gene expression but also several acid-inducible genes including T6SS genes (Wu et al., 2012).

The active research from the late 20th to 21st century has greatly advanced our understandings of the molecular mechanism underlying *Agrobacterium*-mediated transformation. This process can be divided into five major steps as 1) attachment: *A. tumefaciens* cells attach to the plant cell; 2) signal sensing and gene expression: plant signals are sensed by *A. tumefaciens* followed by activation of *vir* gene transcription and generating Vir proteins; 3) T-DNA processing and transport: T-DNA is processed and T-DNA and effector proteins are transported via VirD4/VirB T4SS into plant cells; 4) cytoplasmic trafficking and nuclear import: T-DNA and several effector proteins traffic

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in cytoplasm and enter the nucleus of plant cell; 5) T-DNA integration: T-DNA is integrated into plant genome for tumorigenesis and opine production (Hwang et al., 2017). The key factors involved in each step are briefly described as follows.

Production of cyclic ß-1,2-glucan (Thomashow et al., 1987; Zorreguieta et al., 1988; Cangelosi et al., 1989; O'Connell and Handelsman, 1989), cellulose (Matthysse et al., 1981; Matthysse, 1983) and unipolar polysaccharide (UPP) produced at the pole of an *A. tumefaciens* cell (Tomlinson and Fuqua, 2009) are involved in the attachment of *A. tumefaciens* to the plant cell. Plant or environment signals able to induce the expression of virulence associated genes include phenolic compounds, monosaccharide and acidic pH (Nester, 2014). After expression of virulence-associated genes, T-DNA was cleaved and nicked by endonuclease VirD2 with assistance of VirD1, and the 5' end of the single-stranded T-DNA is bound with VirD2 to form relaxosome (Stachel et al., 1986b; Albright et al., 1987; Jayaswal et al., 1987; Wang et al., 1987; Filichkin and Gelvin, 1993). T-DNA and its associated VirD2 protein and several effectors (VirE2, VirE3, VirF, ORF5) are then recognized by VirD4, a T4SS coupling protein, and transferred independently through T4SS into plant cell (Christie et al., 2014). During T-DNA translocation, T-DNA sequentially interact with T4SS proteins as the order of

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VirD4-VirB11-VirB6/VirB8-VirB2/VirB9 (Cascales and Christie, 2004). It is generally believed that VirE2, single-stranded DNA binding protein may bind to ssT-DNA-VirD2 complex inside plant cytoplasm to form a mature T-complex, which then enters into the nucleus via nuclear pore (Herrera-Estrella et al., 1990; Howard et al., 1992). The mechanism of T-DNA insertion is controversial (Hwang et al., 2017). Whether non-homologous end joining machinery is required for T-DNA differs from different reports. Recently, it is shown that polymerase-theta-mediated DNA repair may be the key player responsible for T-DNA insertion into plant genome (van Kregten et al., 2016).

4. T6SS in *A. tumefaciens*

Several bacterial species possess more than one T6SS gene clusters in their genome, but only one main cluster was found in the sequenced *A. tumefaciens* strains so far (Boyer et al., 2009; Lin et al., 2013; Huang et al., 2015; Cho et al., 2018). *A. tumefaciens* T6SS was first noticed by a secretome analysis that has identified the secretion of Hcp (Wu et al., 2008), which is named based on its co-regulation of hemolysin protein (Williams et al., 1996). As Hcp is considered as a hallmark of a functional T6SS, each of the genes in the T6SS gene cluster of *A. tumefaciens* C58 were

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subjected to in-frame deletion analysis to determine the genes essential for Hcp secretion (Lin et al., 2013). *A. tumefaciens* strain C58 encodes one T6SS main cluster containing 2 operons, *imp* operon and *hcp* operon, and another orphan *vgrG*-associated operon named as *vgrG2* operon located elsewhere (Figure 1A). It is noteworthy that the T6SS outer membrane protein TssJ found in other bacterial species is not encoded in *A. tumefaciens* genome though it is considered as an essential component of T6SS membrane complex (Durand et al., 2015). TssL and TssM are two inner membrane proteins that form a complex in *A. tumefaciens*, and the ATPase domain of TssM is required for Hcp secretion and therefore proposed to energize T6SS machine assembly (Ma et al., 2009; Ma et al., 2012).

A. tumefaciens T6SS is transcriptionally induced by acid signal via ChvG/ChvI two-component system (Wu et al., 2012). When grown in neutral minimal medium, transcription of *imp* operon is suppressed by a mature periplasmic ExoR protein that binds to ChvG to inhibit the signal transduction of ChvG/ChvI two-component system. While periplasmic ExoR is not stable under acidic environment; the inhibition caused by ExoR is lost and transcription of *imp* operon is induced by ChvG/ChvI. The *hcp* operon is expressed at basal level at neutral pH but also upregulated by acid signal at

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transcriptional level. The acid-induced T6SS transcription is also found in transcriptome analysis (Yuan et al., 2008; Heckel et al., 2014).

Other than transcriptional regulation, T6SS is also post-translationally regulated. In *P. aeruginosa,* TagQRST proteins on the membrane are able to sense the perturbation of the membrane triggering the self-phosphorylation of PpkA followed by phosphorylation of Fha, a forkhead associated domain-containing protein, by PpkA (Hsu et al., 2009; Basler et al., 2013; Casabona et al., 2013). It is proposed that this regulation is associated with an interesting phenotype, so called "tit-for-tat"; *P. aeruginosa* T6SS is efficiently firing only when it is attacked by other T6SS from the target cell (Basler et al., 2013). While PpkA is responsible for phosphorylating Fha and activate T6SS, PppA dephosphorylates Fha and represses T6SS, and this regulation pathway is called "threonine phosphorylation pathway (TPP)" (Mougous et al., 2007). While Fha is the phosphorylation substrate by PpkA in *P. aeruginosa* and *Serratia marcescens* (Fritsch et al., 2013), TssL instead of Fha is phosphorylated by PpkA first found in *A. tumefaciens*, and recently in *Vibrio alginolyticus* (Yang et al., 2018). In *A. tumefaciens,* TssL phosphorylation is required for recruitment of Fha for activation of type VI secretion (Lin et al., 2014). T6SS is also negatively regulated at post-translational level by TagF

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protein in a TPP-independent manner (Silverman et al., 2011; Lin et al., 2018). In *P. aeruginosa*, deletion of *tagF* gene increases Hcp secretion, and it is independent from TPP since Hcp secretion of strains with Fha phosphorylation site substitution mutant is still de-repressed in the *tagF* deletion mutant (Silverman et al., 2011). In *A. tumefaciens*, TagF is fused with PppA (Atu4331). Overexpression of TagF-PppA or TagF domain only in *A. tumefaciens* abolished Hcp secretion. Same as *P. aeruginosa*, it is independent from TPP since TssL is still phosphorylated when T6SS is repressed by TagF overexpression (Lin et al., 2018). Interaction between TagF and Fha is shown to be essential for TagF-dependent repression in both *A. tumefaciens* and *P. aeruginosa* (Lin et al., 2018).

In *A. tumefaciens* strain C58, three toxin-immunity pairs were identified, namely type VI DNase effector and immunity 1 and 2 (*tde1-tdi1*, *tde2-tdi2*) and type VI peptidoglycan amidase effector and immunity, *tae-tai* (Ma et al., 2014) (Figure 1A). Tde1 and Tde2 have been shown to exhibit DNase activity while biochemical activity of Tae as a peptidoglycan amidase has not been reported. All of these toxins have toxicity when expressed by an inducible promoter in bacterial cells while Tde but not Tae toxins exhibits T6SS-dependent interbacterial toxicity (Ma et al., 2014). Previous study

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indicates that Tde1 and Tde2 are translocated via the cognate VgrG proteins, and both of them require adaptor/chaperone, Tap-1for Tde1 and Atu3641 for Tde2, for secretion and interbacterial toxicity (Bondage et al., 2016). Also, the interaction relationship among Tap-1, Tde1 and VgrG1 have been determined; Tap-1 and Tde1 can form a complex independent of VgrG1 but they require each other to be loaded onto VgrG1. Interestingly, though PAAR protein is believed to be critical for T6SS assembly (Shneider et al., 2013; Cianfanelli et al., 2016a), deletion of *paar* gene (atu4352) only reduced but not abolished type VI secretion in *A. tumefaciens* (Bondage et al., 2016). Tae was found to be co-precipitated by Hcp in *A. tumefaciens* although no direct interaction was identified when co-expressed in *E. coli* (Lin et al., 2013). Based on the evidence that Tae secretion is always correlated with Hcp secretion, Tae may be loaded in the lumen of Hcp tube for secretion, like Tse2 in *P. aeruginosa* (Silverman et al., 2013). In this study, I found that Tde effector not only requires cognate VgrG for delivery, Tde effector loading onto VgrG is also required for VgrG secretion. The protein-protein interaction data indicates that *A. tumefaciens* VgrG1 and VgrG2 can interact with TssA, TssF, TssG and TssK. Tde loading onto VgrG enhances the interaction between VgrG and T6SS baseplate protein TssK. I also found that TssK interacts with TssM and this interaction is lost in the absence of Tde. Thus, we proposed

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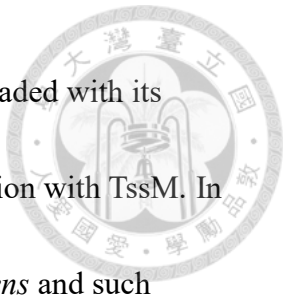

that TssK is able to sense the conformational change of VgrG once loaded with its cognate Tde effector and trigger T6SS machine assembly via interaction with TssM. In summary, our study reveals a novel mechanism found in *A. tumefaciens* and such mechanism may be also conserved in other bacterial species as a strategy to save energy by limiting T6SS machine firing when there is no effector in the cell.

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MATERIALS AND METHODS

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 3. *A. tumefaciens* was grown in 523 medium at 28 ℃ and *E. coli* was cultured in LB medium at 37 ℃ unless indicated otherwise (Lin et al., 2013; Ma et al., 2014; Bondage et al., 2016). The antibiotics were used as the concentrations below. Gentamycin (Gm): 50 μg/mL for *A. tumefaciens* and 30 μg/mL for *E. coli*. Spectinomycin: 200 μg/mL. Kanamycin: 50 μg/mL. Amipicilin: 100 μg/mL. Chloramphenicol: 150 μg/mL.

Medium preparation

LB medium was prepared with dissolving 25 g of the prepared powder (Becton, Dickinson and Company, Sparks, USA) containing 10 g tryptone, 5 g yeast extract and 10 g sodium chloride and adjusted pH to 7.0 per liter followed by autoclave sterilization.

523 medium was prepared by dissolving 10 g sucrose, 8 g casein enzymatic hydrolysate, 4 g yeast extract, 3 g K_2HPO_4, 0.3 g $MgSO_4.H_2O$ in 1 liter water and the

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pH was adjusted to 7.0 followed by autoclave sterilization (Kado and Heskett, 1970).

I-medium was prepared by dissolving 3 g K_2HPO_4, 1 g NaH_2PO_4, 1 g NH_4Cl, 0.15 g KCl and 9.76 g MES ($C_6H_{13}NO_4S.H_2O$) in 900 mL water (Lai and Kado, 1998). For T6SS induction, the pH was adjusted to 5.5 (Wu et al., 2012). After autoclaving, 100 mL of sterile 20 % glucose, 1 mL of sterile $FeSO_4.7H_2O$ (250 mg/ 100 mL), 1.25 mL of sterile 1 M $MgSO_4.7H_2O$ and 1 mL of sterile 0.1 M $CaCl_2$ were added.

For agar plates, 15 g agar was added to 1 L medium above followed by autoclave sterilization.

DNA preparation

Plasmid DNA was extracted using Presto Mini Plasmids Kit (Geneaid, Taiwan). Polymerase chain reaction (PCR) for colony PCR and plasmid construction involves 2X Ready Mix A (Zymeset) by following manufacturer's protocol. *A. tumefaciens tssK* gene and its upstream ribosome binding site was amplified from pRL-TssK (EML1301) (Table 3) with primers *tssK*_BamHI_F and *tssK*_SalI_R (Table 4) containing restriction sites for BanHI and SalI. The PCR product and pTrc200 vector were double-digested with BamH1 and SalI (New England BioLabs, Ipswich, USA) followed by ligation with

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T4 DNA ligase (New England BioLabs, Ipswich, USA). The plasmid construct was confirmed with colony PCR, enzyme digestion, sequencing and western blot of *A. tumefaciens* cells harboring the plasmid.

Mutant construction

In-frame deletion of *A. tumefaciens* mutants were generated with pJQ200KS suicide plasmid (Quandt and Hynes, 1993) via a double crossover process (Ma et al., 2009). In brief, after transformation by electroporation, transformants were selected with 523 agar plate containing gentamycin without sucrose. The Gm resistant colonies were further cultured in LB broth without Gm overnight followed by serial dilutions and spreading onto 523 agar plates containing 5% sucrose without Gm. Bacterial cells undergoing second crossover are able to survive on plates containing 5% sucrose. The deletion mutants were confirmed by colony PCR and western blot.

Type VI secretion assay

Type VI secretion assay was performed as described (Bondage et al., 2016). In brief, *A.*

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tumefaciens strains were cultured in 523 medium overnight and sub-cultured with OD_{600nm} 0.2 as the initial cell density in I medium (pH 5.5) or 523 medium, depending on the purpose of the experiments, for 6 hours at 25 ℃. After subculture, the supernatant and the bacterial cells were separated by centrifugation with 10,000 g for 10 min. Total cell pellets were adjusted to OD_{600nm} 5 and supernatant was filtered with low protein-binding 0.22 μm sterilized filter units (Milipore, Tullagreen, Ireland). Proteins in the supernatants were precipitated by incubation of 1 mL supernatant with 150 μL of 100% trichloroacetic acid (TCA) and 30 μL of 10% deoxycholic acid (DOC) at 4 ℃ overnight followed by centrifugation at 17,000 g, 15 min at 4 ℃. The resulting protein pellets were solubilized with 10 μL of 1 M tris with original pH. All the samples were added with 4X sodium dodecyl sulfate (SDS) sample loading buffer (SSB) and 1/10 volume of 1 M dithiothreitol (DTT) to result in protein samples in 1X SSB and 0.1 M DTT and boiled for 10 min. The samples were then centrifuged with 10,000 g, 10 mins at 4℃ and the resulting supernatant containing solubilized proteins were subjected to SDS-PAGE and western blot analysis.

Western blot analysis

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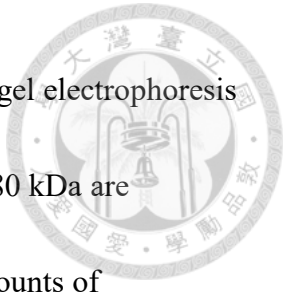

Proteins were usually analyzed with 12 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) except protein whose sizes are under 20 kDa and over 80 kDa are analyzed by 15 % and 7 % SDS-PAGE, respectively. Appropriate amounts of solubilized protein samples were loaded into SDS-PAGE for electrophoresis followed by transferring onto a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane containing protein samples was first blocked with TBST buffer (2.42 g Tris-base, 8 g NaCl, 2 g KCl, and 0.5 mL Tween 20 in liter ddH_2O) containing 5 % skim milk overnight in the cold room, and then changed to TBST containing 5 % skim milk and primary antibody with optimal titer (1:2,500 for Hcp; 1:4,000 for RpoA; 1:4,000 for Tde1; 1:2,000 for Tae; 1:1,000 for VgrG; 1:1,000 for VgrG1; 1:4,000 for TssA; 1:1,000 for TssE; 1:1,000 for TssK; 1:4,000 for TssB; 1:2,000 for ClpV; 1:5,000 for Tap-1; 1:3,000 for HA tag; 1:10,000 for His tag) at room temperature for at least 1 hr (Lin et al., 2013; Bondage et al., 2016). The PVDF membranes were washed with TBST buffer three times, 5 min each, followed by hybridizing with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:20000). Finally, membranes were washed with TBST four times, 5 min each, and Western Lightening ECL Pro Enhanced Chemiluminescence Substrate (PerkinElmer, Watham, USA) was added to produce the chemical luminescence following the user manual. The chemiluminescent was detected

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and visualized with X-ray films.

Co-purification assay in *E. coli*

E. coli co-purification was performed essentially as described previously (Lin et al., 2013). In brief, 5 ml overnight culture of *E. coli* strain BL21(DE3) containing protein expression plasmids was subcultured into 25 ml LB containing appropriate antibiotics for 3 hours and induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) with final concentration of 0.5 mM after subculture for 1 hour. Bacterial cells were collected by centrifugation at 10,000 g, 10 mins and the cell pellet was washed and resuspended with 5 ml lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 15 mM imidazole, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH8.0). Cells were broken with Constant Cell Disruption System (Constant System, Northants, UK) with 40,000 psi. Part of the cell lysate was saved as an input sample. Approximately 1700 μL cell lysate was incubated with 100 μL Ni-NTA resin pre-equilibriumed with lysis buffer for 30 mins at 4 ℃. After binding with the protein, Ni-NTA column containing the specific proteins was washed with 1 ml of wash buffer (50 mM NaH_2PO_4, 0.3 M NaCl, 30 mM imidazole, pH 8.0) four times and the proteins were eluted with 130 μL of elute buffer (50 mM NaH_2PO_4,

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300 mM NaCl, 250 mM imidazole, pH 8.0). Input and elute samples were further analyzed with SDS-PAGE followed by western blot analysis.

Biochemical fractionation of *A. tumefaciens* proteins

Fractionation of *A. tumefaciens* cytoplasmic proteins, periplasmic proteins and membrane proteins was described previously (Ma et al., 2009). In general, 20 ml of overnight cultured *A. tumefaciens* cells in 523 medium was subcultured into 200 ml I medium (pH 5.5) for 6 hours in 25 ℃. Cells were collected and resuspended in 5 mL buffer containing 50 mM Tris-Cl, pH 7.5, 20 % sucrose 2 mM EDTA and 1 mM (PMSF) with addition of lysozyme powder to a final concentration of 0.5 mg/mL and incubated at room temperature with gentle rocking for 1 hour followed by centrifugation at 10,000 g for 10 mins at 4 ℃. The supernatant was saved as periplasmic proteins. Pellets were resuspended in 5 ml of buffer containing 50 mM Tris-Cl (pH 7.5), 0.2 M KCl and 1 mM PMSF. Cells were broken by French Pressure Cell Press (Thermo, Needham Heights, USA) with 40,000 psi and the cytosol and membrane fractions were separated by centrifugation at 150,000 g for 1 hour in micro-centrifuge tube with thicker walls. After centrifugation, the supernatant was collected as cytosol fraction and the

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pellet was solubilized in 50 mM Tris-Cl (pH 7.5) buffer containing 0.2 M KCl and 1 mM PMSF by adding n-dodecyl-β-D-maltoside (DDM) to the final concentration is 1 %. Protein fractions were analyzed by SDS-PAGE followed by western blot analysis.

Co-purification assay in *A. tumefaciens*

A. tumefaciens strain producing His-tagged specific protein expressed on pRL662 was cultured in 10 ml 523 medium overnight and subcultured into 100 ml I medium (pH 5.5) for 6 hours. Same with co-purification experiment in *E. coli*, cells were broken with Constant Cell Disruption System and the proteins were co-purified with a Ni-NTA column. The input and elute samples were analyzed with SDS-PAGE followed by western blot analysis.

Bacterial two hybrid

The bacterial two hybrid assay was conducted following the protocol (Battesti and Bouveret, 2012). *E. coli* strain DHM1 was transformed by heat shock with pT18 and pT25 plasmids expressing adenylate cyclase T18 or T25 domain fused protein. After

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selection of the colonies harboring the two plasmids on LB plates containing chloramphenicol and ampicillin, single colony transformants were cultured in 3 mL LB containing antibiotics and 0.5 mM IPTG. After 30℃ overnight culture, 2 μL of the bacterial culture was dropped onto LB agar plates containing chloramphenicol, ampicillin, 0.5 mM IPTG and 40 μg/mL X-Gal followed by incubation at 30℃ for two days for colorimetric signal development.

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RESULTS

VgrG cargo effector loading is required for cognate VgrG secretion

A. tumefaciens strain C58 encodes two *vgrG* genes and three T6SS toxin-immunity pairs, namely, Tde1-Tdi1, Tde2-Tdi2 and Tae-Tai (Figure 1A) (Lin et al., 2013; Ma et al., 2014). Among the T6SS toxin effectors, Tde1 and Tde2 are DNases and Tae is predicted to be a peptidoglycan amidase. Two VgrG proteins, VgrG1 and VgrG2, are mainly different at the C-terminus, which confers the effector specificity toward Tde1 and Tde2 respectively (Bondage et al., 2016). To determine whether effector loading onto VgrG spike plays any role on the T6SS assembly or firing, type VI secretion assay (Figure 2) to detect the secretion of Hcp and VgrG proteins, a hallmark of a functional T6SS, was conducted in wild type C58 and each of the effector-immunity pair deletion mutants (Figure 1B). The VgrG antibody used in this study is able to detect both VgrG1 and VgrG2, and they can be differentiated with their molecular weight; the upper band is VgrG1 and the lower band is VgrG2 (Lin et al., 2013). As controls, Hcp, VgrG1/2, Tde1, and Tae are secreted from wild type C58 but not from Δ*tssL.* In single, double, and triple toxin-immunity pair mutants, it is interesting to note that VgrG1 secretion is coincided with the secretion of its cognate Tde1 effector (C58 and Δ*tde2-tdi2*).

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Similarly, VgrG2 secretion is only detected from strains capable of Tde2 delivery, i. e. C58, Δ*tde1-tdi1,* Δ*tae1-tai1,* Δ*tae1-tai1*Δ*tde1-tdi1.* Because of low abundance of endogenous cellular Tde2, we are unable to detect Tde2 secretion but Tde2-mediated antibacterial activity has been demonstrated previously (Bondage et al., 2016). While Hcp secretion level is similar to wild type in each of single toxin-immunity pair deletion mutants; surprisingly, no Hcp secretion could be detected in *tde-tdi* double deletion mutant (Δ*tdei*). In other words, Hcp is secreted only when either of the VgrG proteins is secreted. We also found that Δ*tae-tai* mutant has a polar effect as downstream VgrG1, Tap-1, and Tde1 proteins were not detected. As a result, no VgrG1 and Tde1 secretion could be detected whenever *tae-tai* gene pair is deleted. To confirm whether the loss of Hcp and VgrG secretion is indeed caused by the absence of Tde1 and Tde2, complementation test was carried out. Indeed, expression of Tde1 but not Tde2 in Δ*tdei* restored VgrG1 secretion whereas Tde2 but not Tde1 restored VgrG2 secretion (Figure 1C). Hcp secretion is restored whenever VgrG1 or VgrG2 is secreted.

Previous study showed that secretion and/or antibacterial activity of Tde1 and Tde2 require specific adaptor/chaperone proteins for loading onto their cognate VgrG spike for delivery (Bondage et al., 2016). Taken together with the secretion assay results from

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Figure 1B and 1C, we hypothesize that cargo effector loading (such Tde1 onto VgrG1) is not only required for effector delivery but such effector-VgrG interaction may also affect cognate VgrG's function as a T6SS machine component and therefore impact VgrG and Hcp secretion. While the secretion results in various toxin-immunity pair mutants is consistent with our hypothesis, it remains possible that it is the protein accumulation of Tde in the cell affects T6SS machine function rather than the interaction of Tde-VgrG. To rule out this possibility, we conducted secretion assay using T6SS effector adaptor/chaperone deletion mutant, that is Δ*tap-1* and Δ*atu3641* (Figure 3). Tap-1 is a DUF4123-containing protein which is a T6SS adaptor/chaperone protein in both *V. cholerae* and *A. tumefaciens* (Liang et al., 2015; Unterweger et al., 2015; Bondage et al., 2016). In *A. tumefaciens*, Tap-1-Tde1-VgrG1 interaction was demonstrated in our previous study (Bondage et al., 2016); Tde1 and Tap-1 require each other to interact with VgrG1 and Tap-1-Tde1 complex may form first followed by loaded onto VgrG1while the actual interaction interface in the complex is not determined yet. Atu3641, a DUF2169-containing protein, was proposed to be an adaptor/chaperone of Tde2 since there is no Tde2-mediated antibacterial activity when *atu3641* is deleted. Also, co-IP data showed that VgrG2 but not VgrG1 interacts with Atu3641, suggesting that Atu3641 functions as an adaptor/chaperone for Tde2 to be

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loaded onto VgrG2 (Devanand Bondage, unpublished result). As shown in Figure 3, VgrG1 is secreted only in the presence of Tap-1 and *vice versa* for VgrG2 requiring Atu3641 for its secretion. This result excluded the possibility that Tde protein accumulation in the cell may affect T6SS function since Tde effector in these two mutants still accumulates significant amounts in the cell. Taken together, we suggested that cargo effector loading onto cognate VgrG protein is important for VgrG secretion, and this may be a mechanism that *A. tumefaciens* uses to save energy when Tde effector is not loaded onto VgrG.

VgrG proteins interact with baseplate components TssAFGK

To further elucidate the mechanism underlying the requirement of Tde effector loading for VgrG secretion, we went on studying the subcellular localization and the protein interaction network of VgrG. We first co-expressed His-tagged VgrG1 with each of T6SS baseplate components in *E. coli* for co-purification assay (Figure 4 and 5). The results showed that TssA, TssF, TssG and TssK are co-purified with VgrG1 but not TssE, suggesting the direct interaction between VgrG1 with TssA, TssF, TssG and TssK in the absence of other *Agrobacterium* T6SS proteins. TssA-VgrG interaction has been

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demonstrated in both *P. aeruginosa* and EAEC using B2H (Planamente et al., 2016; Zoued et al., 2016). Furthermore, TssF-VgrG and TssG-VgrG interaction is also demonstrated in EAEC with B2H and co-IP (Brunet et al., 2015). However, TssK-VgrG B2H result was negative in EAEC (Zoued et al., 2013).

To confirm the interaction relationship between VgrG and baseplate components, we then used B2H and pulldown assay in *A. tumefaciens* to confirm the interactions. B2H is a quick and sensitive assay for identifying interactions between two proteins (Battesti and Bouveret, 2012) (Figure 6). Thus, we first fused each of baseplate components and VgrG proteins to adenylate cyclase T18 or T25 domain and transformed each pair into *E. coli* strain DHM1 for interaction assay. We are able to detect the TssG-TssK interaction and self-interaction of TssA, TssF, TssG and TssK (Figure 7). However, no interaction activity could be detected for either VgrG self-interaction or interacting with any of tested baseplate proteins even though VgrG-baseplate interaction has been demonstrated for *P. aeruginosa* and EAEC T6SSs with B2H (Brunet et al., 2015; Planamente et al., 2016; Zoued et al., 2016). Thus, we then generated C-terminal His-tagged VgrG1 or VgrG2 to be expressed in *A. tumefaciens* *vgrG* double deletion mutant for co-purification assay (Figure 8). TssA and TssK but not

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TssE were co-purified with VgrG1-His or VgrG2-His. Since His-tagged VgrG remains functional in mediating Hcp secretion (Figure 9A), TssA-VgrG and TssK-VgrG interactions occurs in the context of a functional T6SS (Figure 10A). Co-purification assay of TssK-His in *A. tumefaciens* strain Δ*tssK* also confirmed the TssK-VgrG interaction (Figure 10B), although His-tagged TssK is not functional in mediating Hcp secretion (Figure 9B).

Tde loading enhances VgrG recruitment to the membrane complex and VgrG-TssK interaction

Because TssLM inner membrane complex is proposed as a docking site for baseplate recruitment onto membrane, subcellular fractionation experiment (Figure 11) was conducted to determine VgrG subcellular localization in wild type and Δ*tdei* (Figure 12A). In wild type C58, the inner membrane TssM is only detected in membrane fraction whereas ActC and RpoA soluble proteins are mainly detected in cytoplasmic fractions although RpoA is also present in the membrane fractions, which are 10-fold enriched as compared to cytoplasmic fraction. VgrG proteins are mainly present in cytoplasmic fraction but also detected in membrane fraction in wild type C58. However,

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less VgrG proteins were detected in the membrane fraction of Δ*tdei* as compared to that of C58 wild type and Δ*tssL*. This result suggested that Tde-loaded VgrG has higher affinity or stability in association with membrane, which may be responsible for efficient assembly of T6SS machines on the membrane for abundant secretion of Hcp and VgrG.

Previous cryo-electron microscopy (cryo-EM) reconstruction study in *V. cholerae* proposed that the space between VgrG and TssK in the assembled T6SS machine may accommodate effectors (Nazarov et al., 2017). Thus, it would be interesting to determine whether Tde effector also associates with TssK. Co-purification assay with *A. tumefaciens* Δ*tssK* expressing TssK-His showed significant interactions with VgrG1/2, TssA, and Tde1 and weak interactions with Tap-1 (Figure 10B). Next, we determined whether Tde loading onto VgrG would affect VgrG-TssK interaction. To test this idea, we co-expressed three plasmids for expression of His-tagged VgrG1 from pET28a, TssK from pRL662, and Tap-1, Tde1, Tdi1 and PAAR from pTrc200 in *E. coli* for co-purification of His-VgrG1 via Ni-NTA resin (Figure 12B). Indeed, more TssK protein is co-purified when pTrc200 plasmid harboring *tap-1-tde1-tdi1-paar* is co-expressed compared with the one without co-expression of Tap-1, Tde1, Tdi1 and PAAR. This

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result implied that Tde1 effector loading onto VgrG1 enhances the interaction between VgrG1 and TssK. Since TssK is a baseplate protein interacting with membrane complex protein TssL and TssM in EAEC (Zoued et al., 2013), we then determined whether TssK can interact with TssM or TssL, and if so, whether TssK interaction with Tde loaded VgrG plays any role in the interaction between TssK and the TssL-TssM membrane complex. Thus, TssK-His is expressed in Δ*tssK*, Δ*tdei2*Δ*tssK* (express Tde1 but no Tde2), and Δ*tdei*Δ*tssK* (no Tde1 nor Tde2), for co-purification assay (Figure 13). The results showed that TssM can be pull-downed with TssK via Ni-NTA resin in addition to TssA, TssC, VgrG1/2, and Tde1 in strains harboring both Tde effectors or Tde1 only (Δ*tssK* or Δ*tdei2*Δ*tssK*). When both Tde1 and Tde2 are absent, interaction of TssK with VgrG1/2 and TssM is highly diminished. This result suggested that TssK-TssM interaction only occurs efficiently when VgrG is loaded with Tde cargo effector. Taken together, we proposed a model (Figure 14) that TssK-VgrG interaction is enhanced when VgrG is loaded with Tde effectors and the formation of Tde-VgrG-TssK effector-baseplate complex is required for docking onto TssL-TssM complex. Thus, TssK may function as a connector between VgrG-Tde complex and TssLM membrane complex to initiate baseplate recruitment, polymerization of Hcp and TssBC to assemble into a functional T6SS for secretion.

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Deletion of *tae-tai* in Δ*tdei* background restores Hcp secretion in I medium (pH5.5)

Previous study did not detect significant reduction of Hcp secretion in Δ3TIs, the mutant lacking all three toxin-immunity pair in *A. tumefaciens* C58 (Ma et al., 2014). This seems to conflict with our observation that Tde cargo effector loading is important for cognate VgrG function involved in assembly of a functional T6SS machine. One major difference between the current secretion assays and previous study (Ma et al., 2014) is the different culture media used for the secretion assay. All the secretion assay was carried out in *A. tumefacines* cells grown in acidic minimal medium (I medium) by previous study (Ma et al., 2014) instead of 523 rich medium used in the current study shown in Figure 1 and 3. To determine whether the culture media are the reason for the observed discrepancy, secretion assays for *A. tumefaciens* cells grown in I medium, 523, and LB were carried out. Thus, all *A. tumefaciens* strains were first grown in 523 overnight and the cells were collected for subculture at initial OD_{600nm} 0.2 in different media for 6 hrs. We found that Δ3TIs exhibits wild type level of Hcp secretion when grown in I medium but little Hcp secretion when grown in 523 and LB (Figure 1B and 15). Strikingly, Δ*tdei* (Δ*tde1-tdi1*Δ*tde2-tdi2*) has no or little Hcp secretion in all tested

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media. To determine whether the deficiency of Hcp secretion in Δ*tdei* is due to the presence of *tae* or *tai,* secretion assays were carried out in Δ3TIs with *trans*-expression of *tae* and/or *tai* grown in I-medium pH 5.5 (Figure 16). The data showed that complementation of Tae but not Tai in Δ3TIs is able to abolish Hcp secretion. Thus, we conclude that growth media or stage may regulate type VI secretion and the absence of Tae, a putative peptidoglycan amidase, can bypass the requirement of Tde loading onto VgrG for mediating Hcp secretion, but this phenomenon only occurs when *A. tumefaciens* is grown in I-medium but not in rich media.

VgrG overexpression restores Hcp secretion without Tde loading

Previous study showed that that VgrG C-terminus is required for Tde binding and translocation. This conclusion has been made based on the Tde-dependent antibacterial activity and secretion in a series of truncated VgrG proteins overexpressed in *vgrG* double deleted mutant (Δ*G1G2*) (Bondage et al., 2016). Among the tested truncated VgrG1 proteins, VgrG1^{785} is not able to interact with Tde1 anymore but remains the ability to mediate Hcp and Tae secretion albeit at lower levels in Δ*G1G2* expressing VgrG1^{785}. This result is against the requirement of Tde loading onto VgrG to mediate

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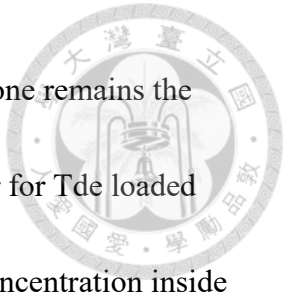

Hcp secretion observed in this study (Figure 1 and 3). Since VgrG alone remains the ability in interacting with TssK albeit the interaction is much stronger for Tde loaded VgrG (Figure 12B and 13), we reason that increased VgrG protein concentration inside the cell may be able to form sufficient amounts of VgrG-TssK complex for interaction with TssLM membrane complex to initiate T6SS assembly and secretion. Thus, I generated the mutant with deletion of *tde1-tdi1*, *tde2-tdi2*, *vgrG1* and *vgrG2* operon and overexpressed full-length or selected truncated VgrG1 variants in this mutant to test whether Hcp can be secreted in the absence of Tde effectors. Indeed, overexpressing full-length VgrG1 and truncated VgrG1 variants ($VgrG1^{812}$, $VgrG1^{804}$ and $VgrG1^{785}$) lacking Tde1 binding domain but remaining Hcp secretion activity reported previously (Bondage et al., 2016) indeed can restore Hcp secretion in Δ*tdei*Δ*G1*Δ*G2op* (Figure 17). As a control, Δ*tdei*Δ*G1*Δ*G2op* alone or overexpression of $VgrG1^{781}$ variant lacking the required domain in mediating Hcp secretion (Bondage et al., 2016) remains deficiency in Hcp secretion. These results suggested that VgrG overexpression can bypass the requirement of Tde loading onto VgrG for assembly of a functional T6SS to mediate Hcp secretion.

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DISCUSSION

T6SS is a versatile machinery possessed by many Gram-negative bacterial species conferring virulence toward eukaryotic host and/or bacterial toxicity to prokaryotic competitors (Russell et al., 2014; Cianfanelli et al., 2016b). T6SS effectors are known to be delivered via being a part of Hcp or VgrG (specialized Hcp or VgrG) or non-covalently bound to Hcp or VgrG (cargo effector) (Cianfanelli et al., 2016b). On the other hand, the baseplate is proposed to dock onto the membrane complex followed by polymerization of Hcp tube and TssBC sheath (Brunet et al., 2014; Brunet et al., 2015; Durand et al., 2015; Vettiger et al., 2017). However, due to the lack of knowledge on the T6SS assembly pathway and often multiple copies of *vgrG* and associated effector genes encoded in a single bacterial genome, it is not feasible to dissect the roles of effector loading on the T6SS activity. In this study, by taking the advantages of only two *vgrG* genes and well characterized effector-VgrG relationship in *A. tumefaciens* strain C58 (Bondage et al, 2016), I have explored the molecular mechanisms underlying how and the impact of Tde effector-VgrG complex is loaded onto T6SS machine. By employment of protein secretion assay and protein-protein interaction studies, the results strongly suggested that the formation of Tde-VgrG-TssK effector-baseplate

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complex is required for docking onto TssL-TssM complex and thus initiate Hcp polymerization and TssBC assembly into a functional T6SS for secretion.

VgrG interacts with baseplate components TssA, TssF, TssG, and TssK

Previous studies have suggested that VgrG interacts with several baseplate components and such interactions may be critical for assembly of T6SS (Brunet et al., 2015; Planamente et al., 2016; Zoued et al., 2016). However, it is not known whether or which baseplate components interact with VgrG in *A. tumefaciens*. With *E. coli* co-purification assay, we identified four baseplate proteins interacting with VgrG, that is TssA, TssF, TssG and TssK but not TssE (Figure 4). Interaction between VgrG and TssAK was further confirmed in *A. tumefaciens* cell with co-purification assay with C-terminal His-tagged VgrG or TssK (Figure 10). Interestingly, VgrG but also Tap-1 and Tde1 are pull-downed by His-tagged TssK, suggesting that TssK interacts with VgrG-Tde complex. While Tap-1 could be pull-downed with TssK, the interaction is relatively weak, unlike the strong interaction between TssK and VgrG1 or Tde1. Since Tap-1 is required for Tde1 loaded onto VgrG1 but itself is not a T6SS substrate (Liang et al., 2015; Bondage et al., 2016), Tap-1 may only transiently interact with VgrG1 and may

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fall out from the complex when Tde1 effector is loaded onto VgrG1-baseplate complex for T6SS machine assembly.

The roles of Tde loading for T6SS subcomplex assembly and VgrG/Hcp secretion

We demonstrated the importance of VgrG cargo effector loading on the secretion of VgrG by performing secretion assay in various mutants including effector-immunity pair deletion mutants and T6SS effector adaptor/chaperone deletion mutants (Figure 1 and 3). In order to dissect the role of Tde-Tdi toxin-immunity pair for VgrG secretion, I first used His-tagged VgrG pull-down assay in Δ*tdei*Δ*vgrG1*Δ*vgrG2 operon* to understand the underlying mechanism. However, overexpression of either full-length or truncated VgrG1 variant lacking the Tde1 interaction domain (VgrG1^{785}) could restore Hcp secretion (Figure 17). This phenomenon is consistent with the observation that overexpression of VgrG1^{785} in *vgrG1vgrG2* double deletion mutant is able to restore Hcp secretion though there is no any Tde loading in this situation (Bondage et al., 2016). These results are conflicting with our observation for the requirement of Tde loading onto cognate VgrG for mediating the VgrG secretion. Since VgrG can interact with TssK in the absence of its cognate Tde effector in both *E. coli* and *A. tumefaciens*,

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it is possible that high concentration of VgrG proteins in the cytosol can cause sufficient interaction with baseplate and thus force T6SS machine to assemble even VgrG is not loaded with cognate Tde effector. Indeed, such notion could be supported with the results of the biochemical fractionation, *E. coli* co-purification analysis with co-expression of Tap-1, Tde1, Tdi1 and PAAR, and *A. tumefaciens* TssK co-purifcation assay in effector-immunity mutants (Figure 12B and 13). The results suggest that VgrG-TssK interaction is enhanced when Tde is loaded onto cognate VgrG and interaction between TssK and Tde-loaded VgrG is required for TssK-TssM interaction. Thus, TssK may have higher affinity in interacting with Tde loaded VgrG than VgrG alone and only efficiently trigger the assembly of T6SS machine when Tde effector is loaded onto VgrG (Figure 13). Our secretion assay results indicate that C-terminal His-tag probably affect the function of TssK as Hcp secretion in *tssK* deletion mutant expressing TssK-His is not restored (Figure 9B). It is possible that the C-terminal tag affects the function of TssK after T6SS baseplate docking onto TssLM membrane, since our TssK-His protein is still able to interact with known interacting proteins including TssM, TssA and TssC (Zoued et al., 2013). Therefore, the interaction results may remain valid in dissecting the initial Tde-VgrG docking mechanisms prior to Hcp tube polymerization. Future work to carry out co-IP using specific α-TssK antibody or generating secretion

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competent epitope-tagged TssK for co-purification assay will be critical to confirm the role of TssK in recruiting Tde-loaded VgrG onto TssLM membrane complex for assembly of a functional T6SS.

Because VgrG secretion is abolished in the absence of its cognate effector or adaptor/chaperone required for Tde effector loading onto cognate VgrG (Figure 1 and 3) and Hcp secretion is also abolished in the absence of both Tde1 and Tde2 (Figure 1B and 1C), we propose that cargo effector loaded onto VgrG is important for efficient assembly of T6SS machine. This notion is indeed also supported by previous studies in *V. cholerae* as deletion of *vasX* effector and *vgrG1* or *vgrG3* strongly decrease Hcp secretion and sheath assembly but sheath assembly remain almost the same when only *vgrG1* or *vgrG3* is deleted, indicating effector loading plays a role in T6SS assembly through an unknown mechanism (Dong et al., 2013; Vettiger and Basler, 2016). In *A. tumefaciens* strains, 1D1108, 12D1 and 15955, which only harbor one main T6SS gene cluster with only one *vgrG* gene, deletion of *vgrG* downstream putative cargo effectors also caused diminished Hcp secretion (Chih-Feng Wu, unpublished data). Thus, this may be a conserved mechanism and strategy deployed by many T6SS possessing bacteria to save energy when there is no effector in the cell. VgrG cargo effector loading

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is likely to affect at the step where VgrG interacts with baseplate, an important step for initiating the Hcp polymerization and TssBC sheath assembly for T6SS firing (Brunet et al., 2015). Since there is less VgrG protein loaded onto the membrane of *tde-tdi* double deletion mutant of C58 and TssK-TssM interaction is also lost in this mutant (Figure 12A and Figure 13), Tde loading onto VgrG is likely required for efficient interaction with baseplate component TssK, and formation of Tde-VgrG-TssK complex is required for baseplate complex docking onto TssLM membrane.

Protein-protein interaction study of VgrG and baseplate components reveals interaction between VgrG and TssAFGK. VgrG-TssA interaction is already found in both EAEC and *P. aeruginosa* although the TssA protein in these two bacteria is quite different from each other (Planamente et al., 2016; Zoued et al., 2016). Interaction between TssFG and VgrG is also demonstrated in EAEC (Brunet et al., 2015). This is the first time that VgrG-TssK interaction is identified. From the cryo-EM reconstruction image of T6SS baseplate of *V. cholerae*, and the structure of a bacteriophage T4 baseplate, TssK or TssK homolog (gp10 or gp8) is the outermost baseplate component (nearest to the membrane complex) compared with other baseplate components TssAEFG and the space between VgrG and TssK is proposed to accommodate VgrG

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cargo effectors (Taylor et al., 2016; Nazarov et al., 2017). In EAEC, TssK is found to be a homolog of receptor binding protein of siphophage and evolved to connect baseplate and membrane complex in EAEC T6SS (Nguyen et al., 2017). It is also proposed that TssK may be the first baseplate protein recruited to the membrane complex during T6SS machine assembly (Brunet et al., 2015). Similar to *V. cholerae* and T4 phage, TssK is the outermost baseplate protein in EAEC. From the cryo-EM reconstruction and the crystal structure of VgrG protein, it is proposed that VgrG is located in the baseplate with C-terminus toward the membrane (Shneider et al., 2013; Bock et al., 2017; Chang et al., 2017; Nazarov et al., 2017; Nguyen et al., 2018). Therefore, TssK is likely to be the baseplate component responsible for sensing VgrG cargo effector loading and trigger T6SS machine assembly. Our results are consistent with this idea since TssK-VgrG interaction is enhanced when Tde is loaded onto VgrG and this conformation is favored for TssK-TssM interaction thus the T6SS machine is further assembled and contracted for VgrG, Hcp, and effector secretion. On the contrary, when Tde is not loaded, TssK-VgrG interaction is reduced and T6SS machine assembly is limited by losing of TssK-TssM interaction (Figure 12 and 13).

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The roles of *tae* in Hcp secretion in different culture media

Previously, Ma et al (2014) showed wild-type levels of Hcp secretion in Δ3TIs, a mutant with all three effector-immunity gene pairs deleted. This result is contradictory to our observation that Tde loading is required for T6SS secretion. We noticed that one major difference between these two studies is the growth medium. While Hcp secretion is active in *A. tumefaciens* grown in both 523 rich medium (pH 7.0) and acidic minimal medium (I medium pH 5.5) (Wu et al., 2008), VgrG is more readily detected only when grown in rich medium such as 523 but not in I medium (Chih-Feng Wu, unpublished results). Thus, we have chosen 523 medium for the purpose of detecting VgrG secretion and accidentally found that Hcp secretion is highly diminished when *A. tumefacines* Δ3TIs is grown in 523 medium in contrast to previous observation that Hcp secretion is normal from this mutant grown in I medium (pH5.5) (Ma et al., 2014). To determine whether the different Hcp secretion levels observed in Δ3TIs are due to the different culture media used, type VI secretion assay was also carried out in *A. tumefaciens* strains grown in I medium (pH5.5) as well as LB, another rich medium, (Figure 15). Indeed, wild-type level of Hcp secretion was observed from Δ3TIs grown in I medium (pH5.5) but only trace amounts of Hcp secretion were detected in this mutant when

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grown in LB (Figure 15B), similar to what was observed for growth in 523 (Figure 1B). Similar results were also observed for Δ*tde2-tdi2*Δ*tae-tai* because of polar effect of *tae-tai* deletion, in which downstream gene products including VgrG1 and Tde1 were not detectable in any mutant with deletion of *tae-tai* gene pair. Thus, Δ3TIs and Δ*tde2-tdi2*Δ*tae-tai* exhibit similar secretion outcomes in all growth conditions. Such effect is caused by the presence of *tae* locus and likely Tae protein because Hcp secretion is strongly diminished in Δ3TIs complemented with trans-expression of *tae* but not *tai* (Figure 16).

Tae is known to interact with Hcp (Lin et al., 2013), suggesting it may be located inside Hcp tube lumen during T6SS assembly (Silverman et al., 2013; Whitney et al., 2014). Thus, it is possible that the loading of Tae into Hcp hexamer/tube may have an impact in Hcp polymerization and length of Hcp tube as well as surrounding TssBC sheath. In T6SS, nothing is known about how Hcp length is controlled. While T6SS structural components are highly analogous to those of T4 phage, tape-measuring protein and capping proteins are missing in T6SS (Taylor et al., 2018). Tape-measuring protein functions to control the length of the sheath and capping protein is able to prevent the tube from sliding out during sheath contraction (Abuladze et al., 1994;

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Leiman et al., 2004). TssA may function as a capping protein as TssA in EAEC located at distal end of TssBC sheath (Zoued et al., 2016; Zoued et al., 2017). However, *A. tumefaciens* TssA is quite different from both EAEC TssA and *P. aeruginosa* TssA1 (Planamente et al., 2016; Zoued et al., 2016; Zoued et al., 2017). Interestingly, ultra-long T6SS sheathes were observed in ampicillin treated enlarged *V. cholerae* cells (Vettiger et al., 2017) and a unique T6SS encoding tape-measure protein was found in amoebae parasites *Amoebophilus asiaticus* possessing narrow sheath length distribution (Bock et al., 2017). Based on our observation of the effects of Tae in Hcp secretion amounts of *A. tumefaciens* grown in I-medium (pH 5.5), we proposed that Tae may function as a tape-measuring protein or capping protein to control the length of Hcp tube and TssBC sheath for firing. In I medium pH5.5, when Tae protein is missing inside Hcp tube, there may be no control of Hcp tube and produce ultra-long Hcp tube, which then results in higher level of Hcp secretion in Δ3TIs as compared to almost no secretion in Δ*tdei*.

However, it should be noticed that the effect of Tae on Hcp secretion levels is only evident when *A. tumefaciens* is grown in I medium (pH 5.5), a minimal medium that *A. tumefaciens* has a longer doubling time (2.5-4 hr) as compared to shorter doubling time

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(~1.5-2 hr) when grown in 523 rich medium (Morton and Fuqua, 2012). The longer doubling time in I medium may allow the bacterial cell to build longer Hcp tube as opposed to shorter Hcp tube in the cell grown in rich medium before firing. As a result, longer Hcp tube may be only evident in the absence of Tae when grown in minimal medium but not in rich medium. Since Hcp secretion levels are contributed by the number of assembled T6SS, speed/frequency of T6SS contraction/firing, and length of Hcp tube, wild-type level of secreted Hcp amounts in Δ3TIs could be caused by lower number of assembled T6SS machine but increased length of Hcp tube. However, the platform to visualize T6SS contracting foci and Hcp tube/TssB sheath has not been established in *A. tumefaciens*. Future work to develop such experimental platform is the key to unravel the mechanisms of factors regulating T6SS assembly and firing.

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CONCLUSIONS AND FUTURE PERSPECTIVES

T6SS is a nanomachine evolutionally and structurally similar to bacteriophages and T6SS effectors are known to have diverse biological functions so far (Cianfanelli et al., 2016b; Lien and Lai, 2017; Taylor et al., 2018). Though T6SS effectors are usually not regarded as a component of T6SS machine, we found that effector loading onto VgrG indeed plays a role to the T6SS assembly. This phenomenon may be implied as a strategy that agrobacteria use to save energy when there are no effectors in the cell since Tde effectors are major weapons contributing to T6SS-mediated antibacterial activity in *A. tumefaciens* (Ma et al., 2014). According to the protein-protein interaction results, agrobacteria likely prevent T6SS machine assembly via interaction between TssK and VgrG. In the absence of effector loading, interaction between TssK and VgrG is weaker as compared with the one with effector loading and the interaction between TssK and TssM is lost. Thus, baseplate is likely not recruited to T6SS membrane complex and therefore there is no T6SS machine assembly when VgrG is not loaded with Tde. Our results suggest a novel mechanism used by agrobacteria or all the T6SS possessing bacteria to limit energy consumption by avoiding T6SS firing without VgrG cargo effector.

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On the other hand, Hcp interacting effector, Tae, was found to have function to control T6SS in a manner different from VgrG cargo effectors, Tde1 and Tde2. We proposed that Tae possibly functions to control the length of T6SS sheath or prevent the sliding out of Hcp tube.

In the future, it would be interesting to use microscopy technique to understand what is going on during *A. tumefaciens* T6SS assembly and determine the function of Tae during T6SS machine assembly or firing.

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FIGURES

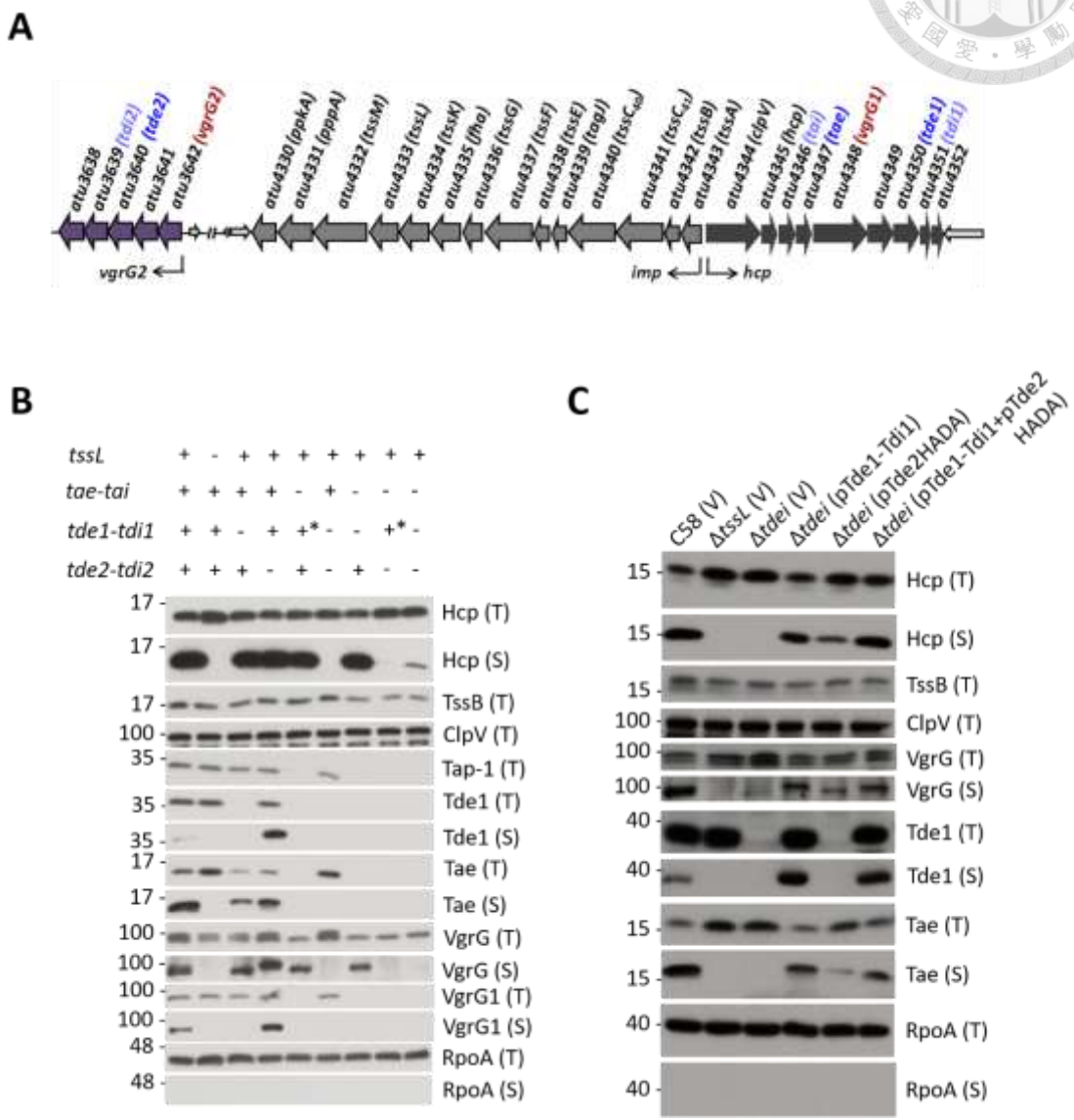

Figure 1. T6SS gene cluster and the effects of toxin-immunity gene pairs in Hcp and VgrG secretion. (A) T6SS gene cluster in *A. tumefaciens* C58. (B) Secretion assay of *A. tumefaciens* wild type strain C58 (positive control), various single, double, and triple toxin-immunity gene pair mutants, and Δ*tssL* (negative control). (C) Secretion assay of

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tde double deletion mutant (Δ*tdei*) containing vector (V) only or expression of single or double *tde* effector genes. Total (T) and secreted (S) fractions were collected from *A. tumefaciens* strains grown in 523 liquid medium for western blotting analysis using various antibodies as indicated. Both VgrG1 (upper band) and VgrG2 (lower band) can be detected by α-VgrG antibody against purified VgrG1-His proteins while VgrG1 but not VgrG2 can be recognized by α-VgrG1 antibody against synthetic VgrG1 C-terminus (Bondage et al., 2016). Asterisk: *tae-tai* deletion has polar effects in downstream protein Tde1 accumulation (see details in text). Non-secreted RpoA protein serves as an internal control. Molecular weight markers (in kDa) were indicated on the left.

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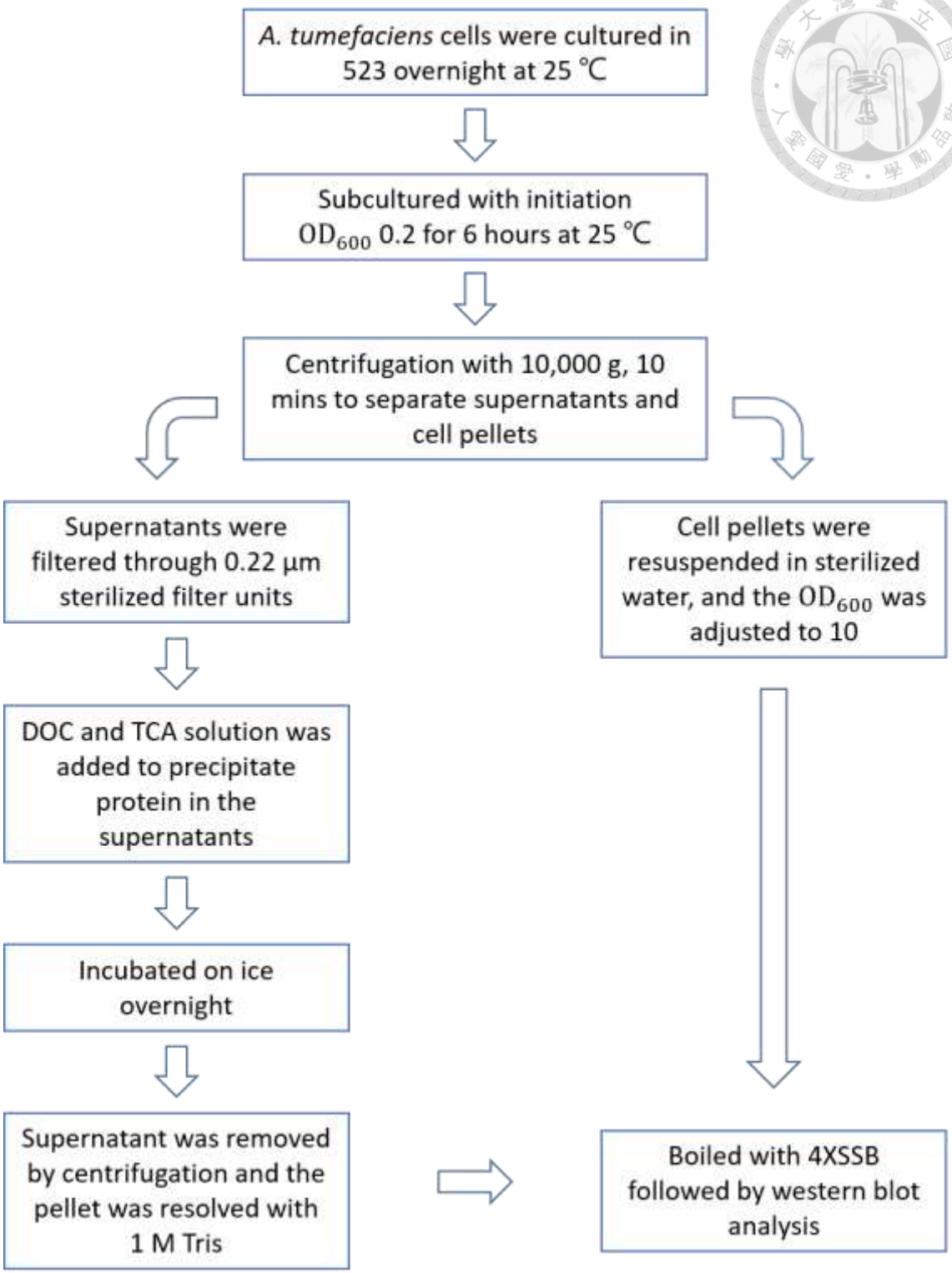

Figure 2. The experimental design and the flow chart of type VI secretion assay.

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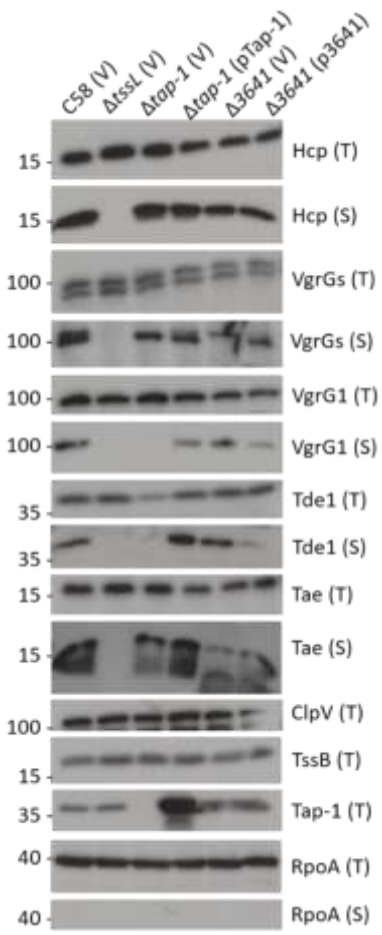

Figure 3. Effects of Tap-1 and Atu3641 in VgrG1 and VgrG2 secretion. Secretion assay of *A. tumefaciens* wild type C58, Δ*tssL,* Δ*tap-1,* and Δ*atu3641* harboring vector (V) only or complementation plasmid. Total (T) and secreted (S) fractions were collected from *A. tumefaciens* strains grown in 523 liquid medium for western blot analysis using various antibodies as indicated. Molecular weight markers (in kDa) were indicated on the left.

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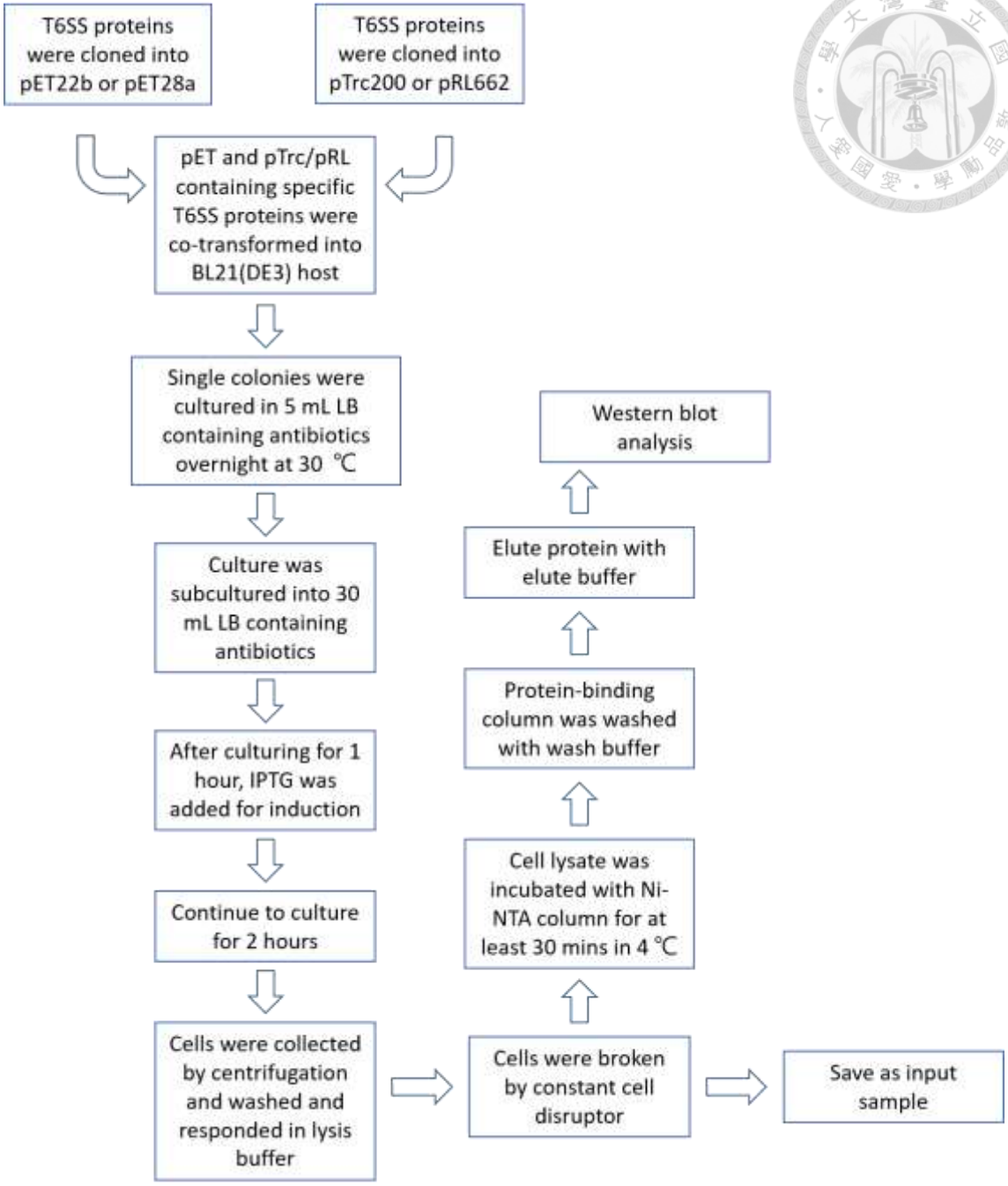

Figure 4. The experimental design and the flow chart of *E. coli* co-purification assay.

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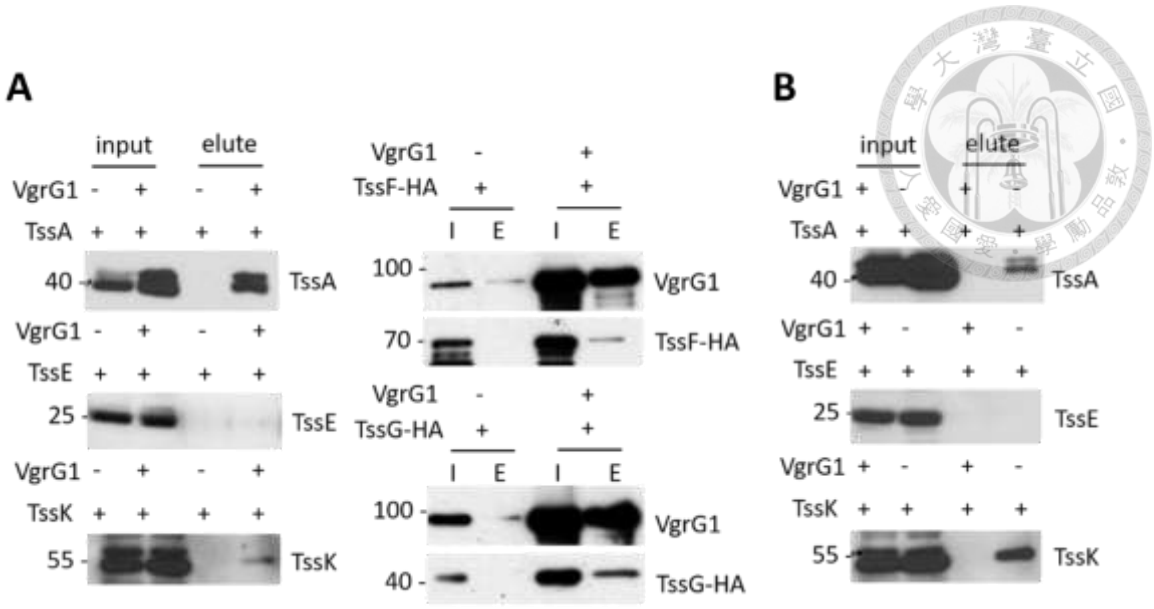

Figure 5. *E. coli* co-purification assay for interactions between VgrG1 and baseplate components. Each of baseplate components is expressed on pTrc200 (TssA, TssE, TssK) or pRL662 (TssF-HA and TssG-HA) plasmids. VgrG1 is expressed on pET28a or pET22b for N-terminal or C-terminal His-tag, respectively. (A) Co-purification in *E. coli* strain BL21 (DE3) using N-terminal His-tagged VgrG1. (B) Co-purification in *E. coli* strain BL21 (DE3) using C-terminal His-tagged VgrG1. Both inputs and eluted fraction from Ni-resin were analyzed by western blot using various antibodies against. VgrG1, TssA, TssE, and TssK were detected by its specific antibody and TssG-HA and TssF-HA were detected by α-HA antibody. Molecular weight markers (in kDa) were indicated on the left.

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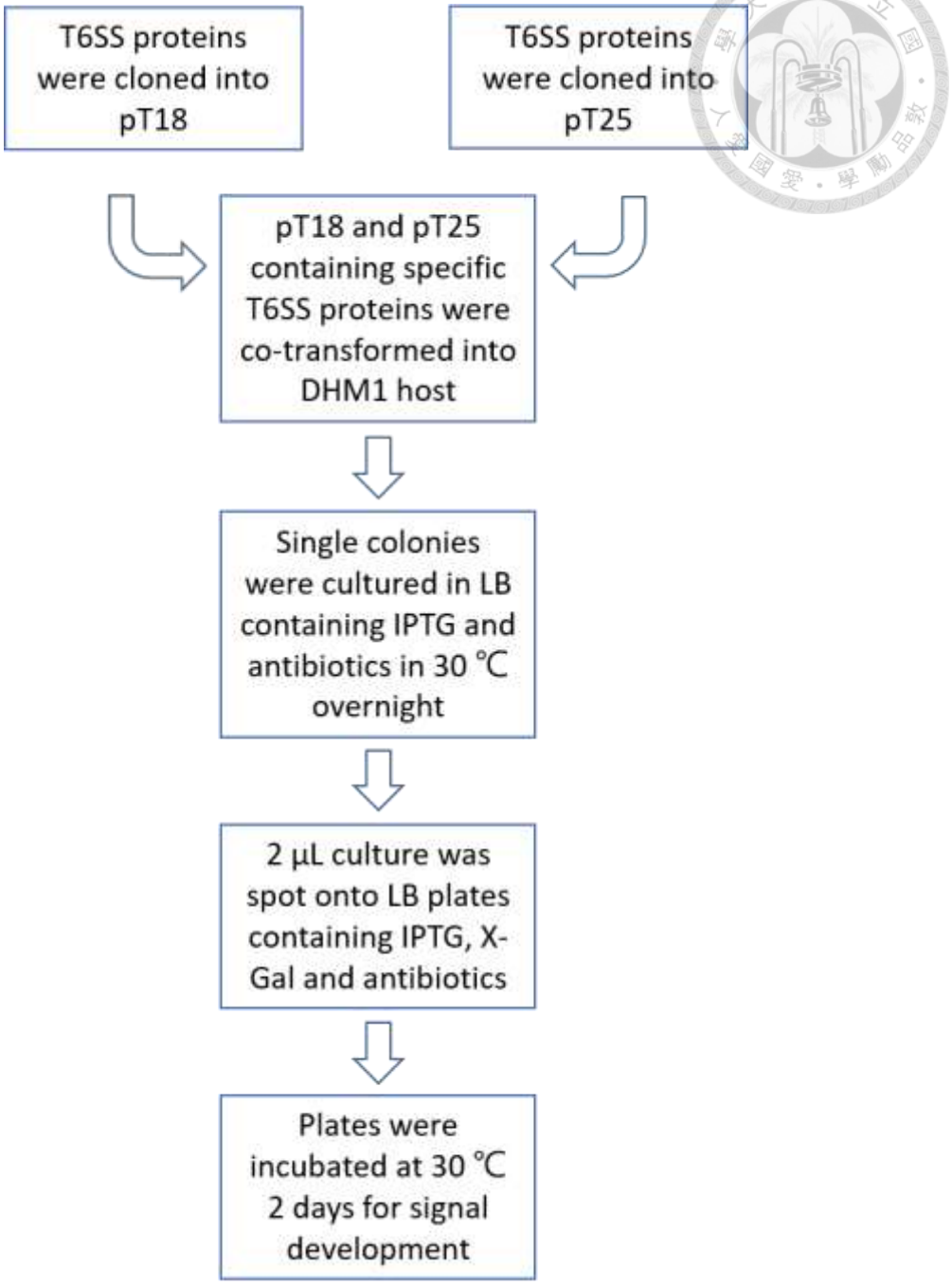

Figure 6. The experimental design and the flow chart of bacterial two-hybrid assay

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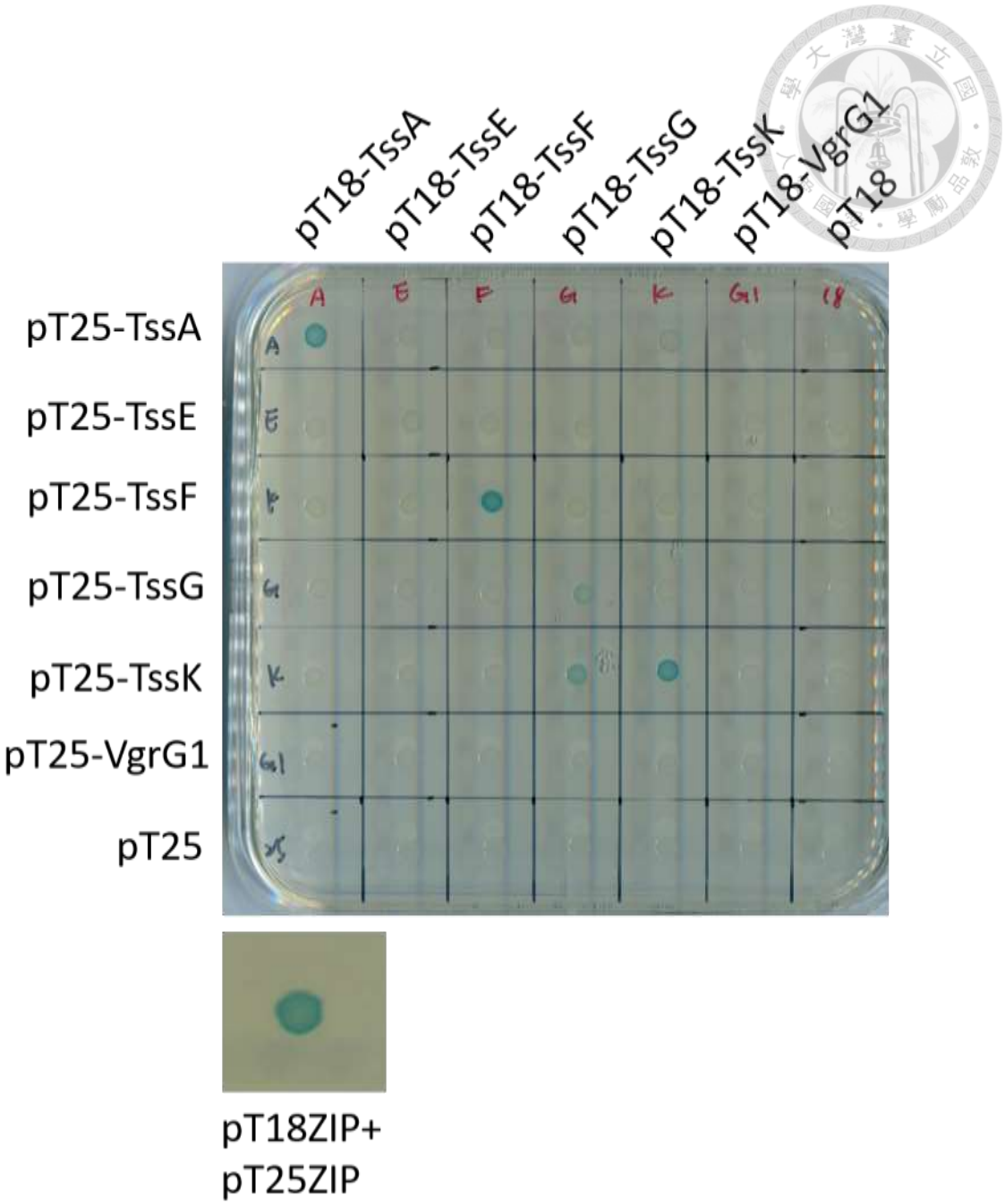

Figure 7. Bacterial two hybrid analysis of TssAEFGK baseplate components and VgrG1. Each protein is fused to T18 (marked in red) and T25 (marked in black) domains respectively and tested for their pairwise interactions by co-transformation into

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E. coli DHM1. The *E. coli* DHM1 strain harboring pT18ZIP and pT25ZIP was used as a positive control, and the strain containing pT18 and pT25 vectors was used as a negative control.

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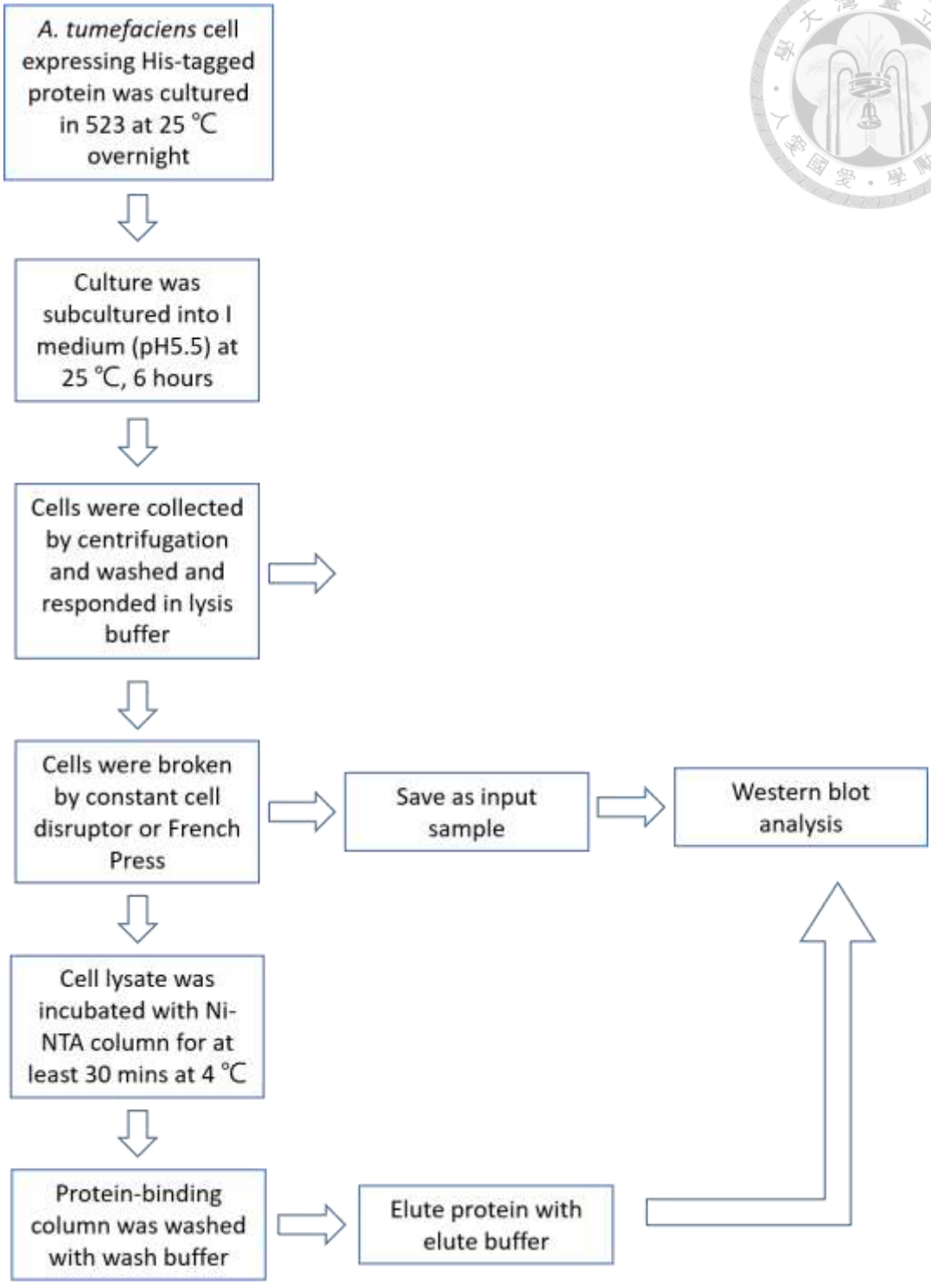

Figure 8. The Experimental design and the flow chart of *A. tumefaciens* co-purification assay by His-tagged protein.

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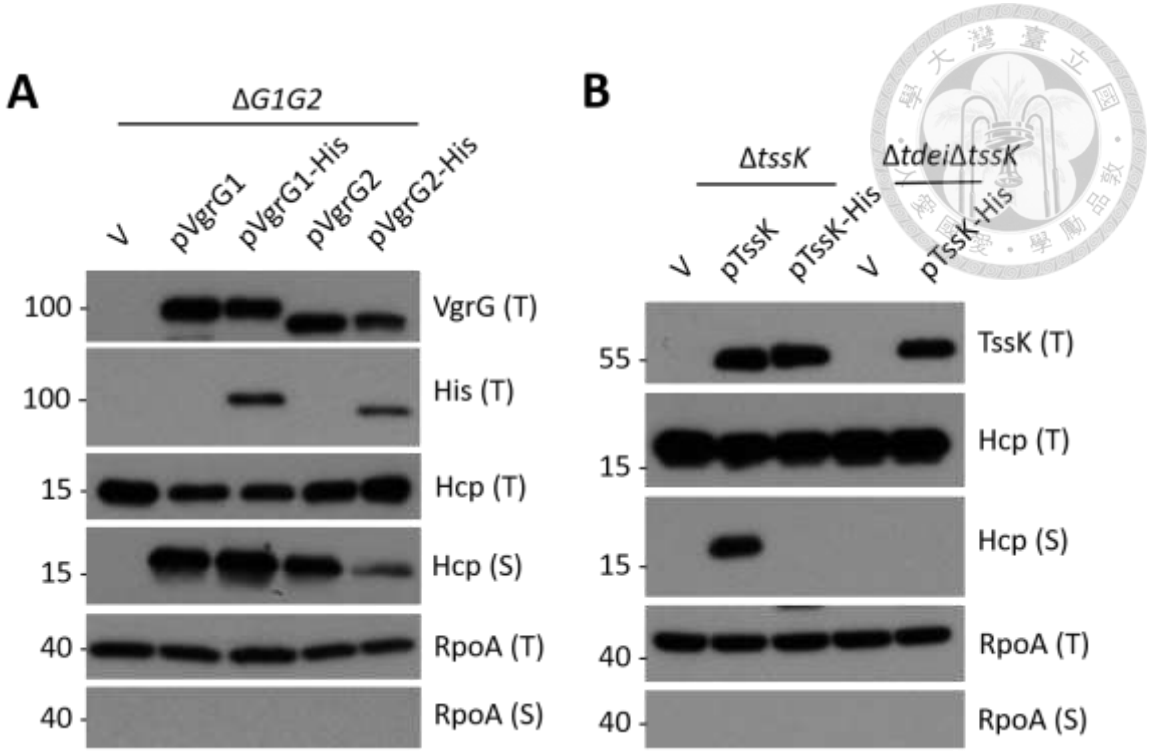

Figure 9. Functional assay of His-tagged proteins used in this study. (A) C-terminal His-tagged VgrG proteins are functional for T6SS secretion. Secretion assay was done in I medium pH5.5 using *vgrG* double deletion mutant complemented with wild type VgrG or C-terminal His-tagged VgrG proteins. (B) C-terminal His-tagged TssK is not functional for T6SS secretion. Secretion assay was done in I medium pH5.5 using tssK deletion mutant or *tdei* double deletion mutant with additional deletion of *tssK* complemented with wild type TssK or C-terminal His-tagged TssK. Molecular weight markers (in kDa) were indicated on the left.

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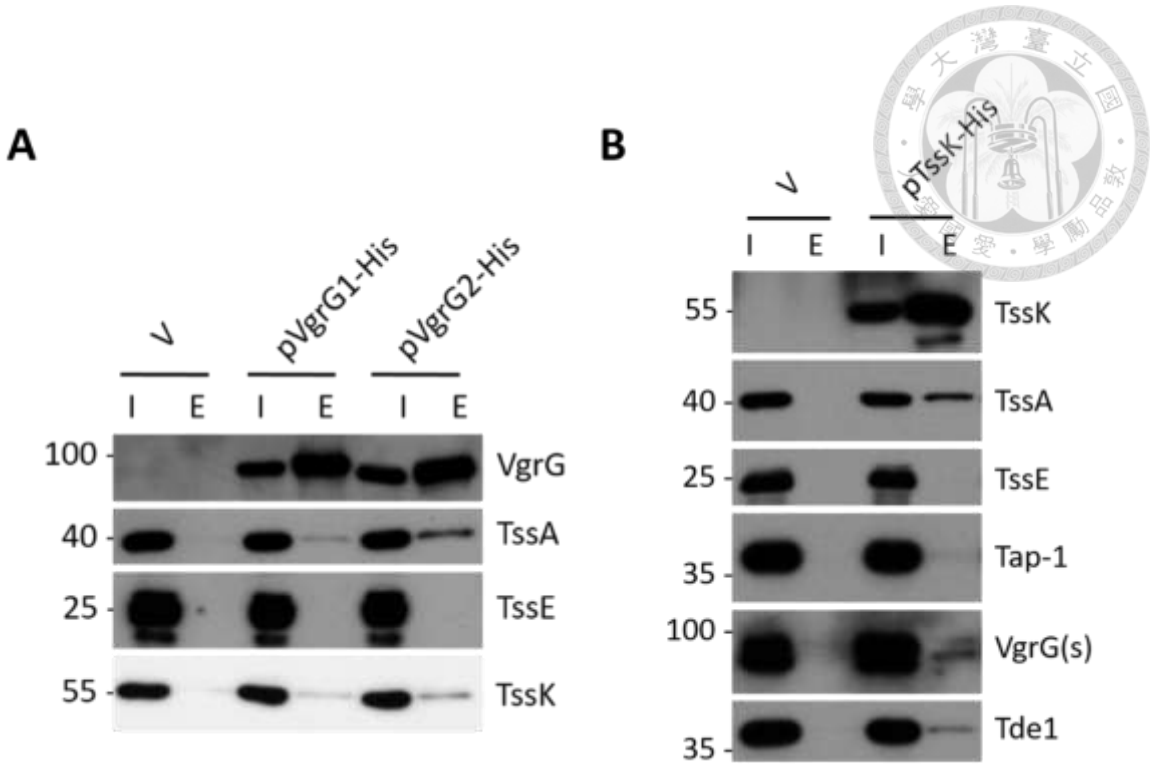

Figure 10. Co-purification assay of His-tagged VgrG1/2 and TssK in *A. tumefaciens*. (A) *A. tumefaciens* Δ*vgrG1*Δ*vgrG2* mutant harboring pRL662 (V) or its derivative expressing C-terminal His-tagged VgrG1 or VgrG2 grown in I-medium is used for pull-down assay by Ni-NTA column. Both input and eluted fractions were analyzed by western blot for detection of VgrG1/2, TssA, TssE, and TssK using specific antibodies. (B) *A. tumefaciens* Δ*tssK* mutant harboring pRL662 (V) or its derivative expressing C-terminal His-tagged TssK (TssK-His) is used for pull-down assay by Ni-NTA column. The input and eluted fractions were analyzed by western blotting for detection of TssK, TssA, TssE, Tap-1, VgrG1/2, and Tde1 using specific antibodies. I: input, cell lysate

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before Ni-NTA column purification. E: elute, eluted fraction after purification.

Molecular weight markers (in kDa) were indicated on the left.

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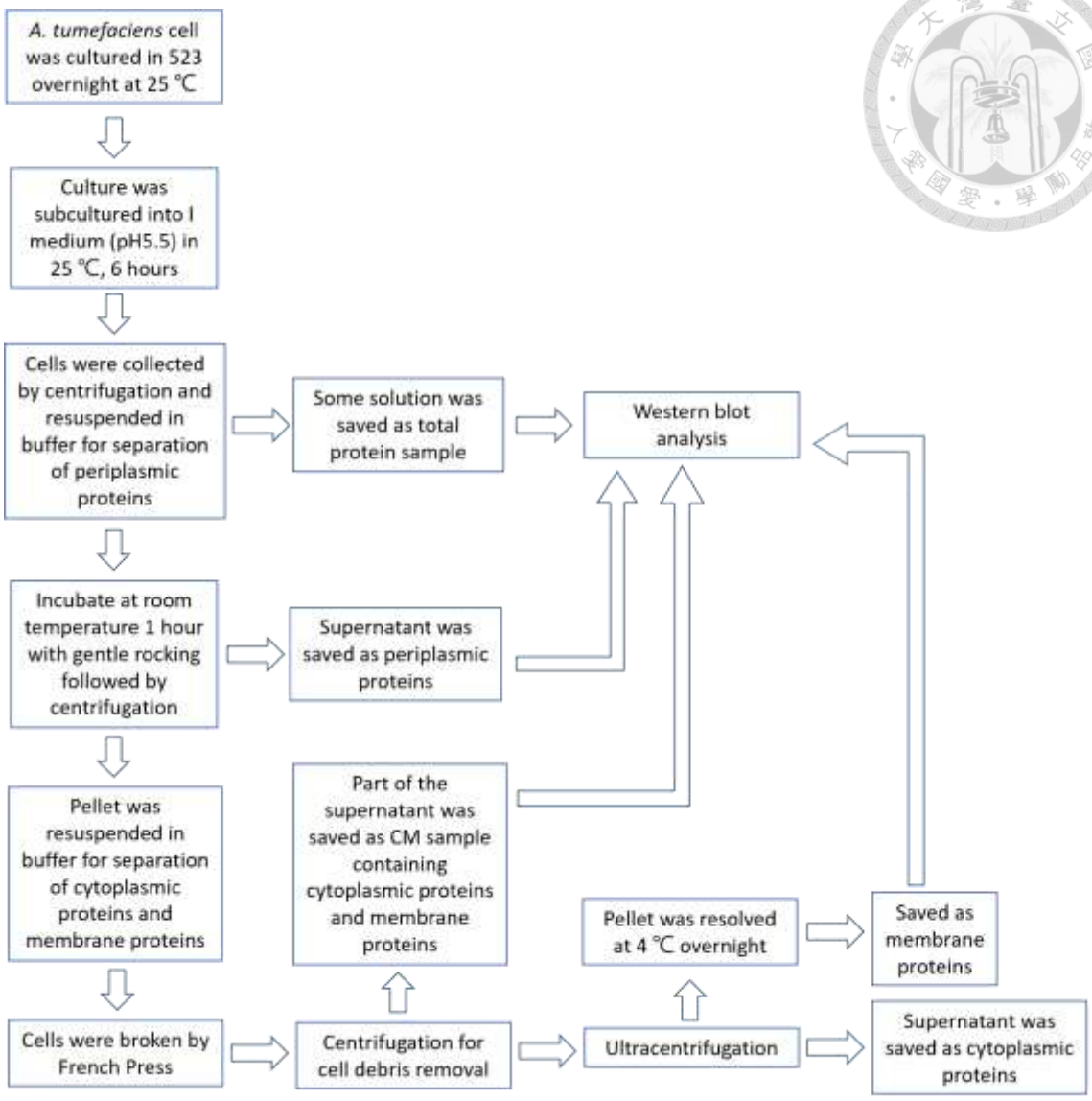

Figure 11. Experimental flow chart of subcellular fractionation.

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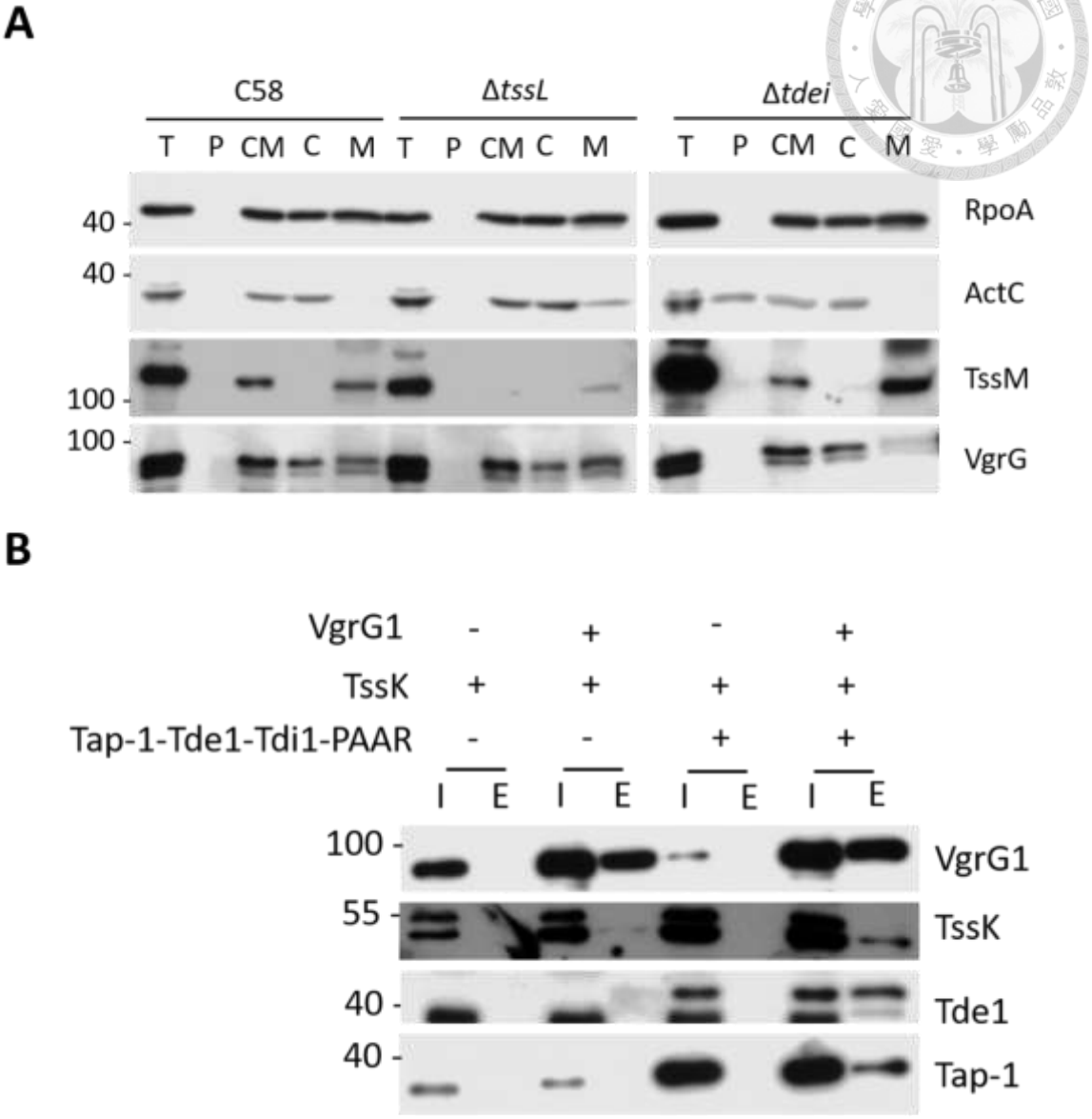

Figure 12. Effects of Tde in amounts of membrane-associated VgrG and VgrG-TssK interaction. (A) *A. tumefaciens* C58 wild type, Δ*tssL* and Δ*tdei* strains were grown in I-medium and subjected for subcellular fractionations. Each fraction is loaded normalized from the same number of cells except membrane fraction is loaded with 10-fold

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amounts for western blotting using specific antibodies. T: total protein. P: periplasmic protein. CM: cell lysate before separation of cytoplasmic fraction and membrane fraction. C: cytoplasmic fraction. M: membrane fraction. ActC, RpoA and TssM are used as markers for periplasmic fraction, cytoplasmic fraction, and membrane fraction, respectively. (B) TssK is co-expressed in the presence or absence of His-VgrG1 and/or pTrc200 harboring *tap-1-tde1-tdi1-paar* in *E. coli* BL21(DE3) strain for co-purification by Ni-NTA column. The input and eluted fractions were analyzed by western blot for detection of VgrG1, TssK, Tap-1, and Tde1 using specific antibodies. Tap-1, Tde1, Tdi1 and PAAR are expressed on pTrc200. His-VgrG1 and TssK are expressed on pET28a and pRL662, respectively. I: input, cell lysate before Ni-NTA column purification. E: elute, eluted fraction after purification. Molecular weight markers (in kDa) were indicated on the left.

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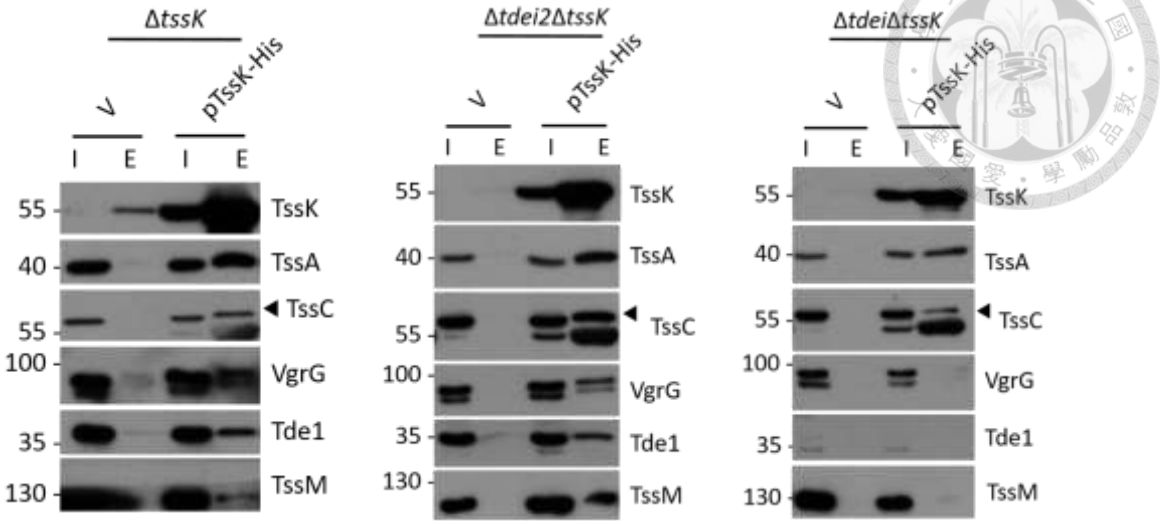

Figure 13. *A. tumefaciens* co-purification assay of Δ*tssK,* Δ*tdei2*Δ*tssK* (*tde2-tdi2* deletion mutant with additional deletion of *tssK*)*,* or Δ*tdei*Δ*tssK* (*tde1-tdi1* and *tde2-tdi2* double deletion mutant with additional deletion of *tssK*) harboring pRL662 (V) or its derivative expressing C-terminal His-tagged TssK grown in I-medium is used for pull-down assay by Ni-NTA column. The input (I) and eluted fractions (E) were analyzed by western blotting for detection of TssK, TssA, TssC, VgrG1/2, Tde1, and TssM using specific antibodies. Molecular weight markers (in kDa) were indicated on the left.

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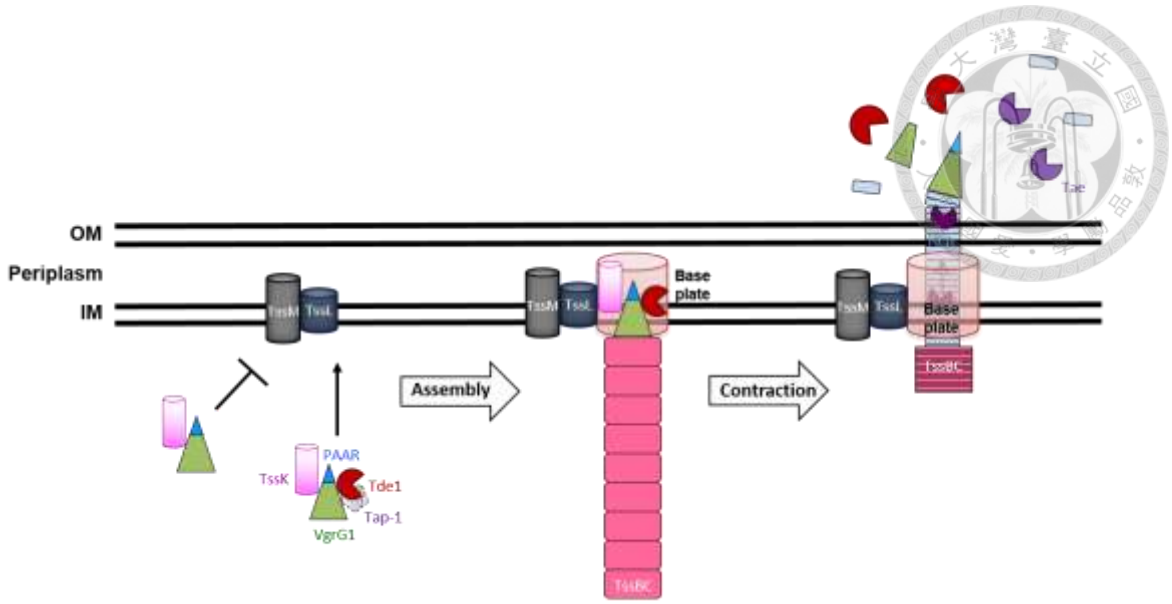

Figure 14. Proposed model of Tde loading onto VgrG in T6SS assembly. In wild type C58, Tde1/2 is loaded onto VgrG1/2 respectively and VgrG-Tde-TssK complex is recruited to TssLM membrane complex, followed by recruitment of other baseplate proteins Tss(A)EFG, and initiation of Hcp polymerization, TssBC assembly and contraction for T6SS secretion. In the absence of Tde effectors or respective adaptor/chaperone, unloaded VgrG may weakly interact with TssK and this VgrG-TssK complex is not recruited onto TssLM membrane complex and therefore the T6SS assembly is abolished.

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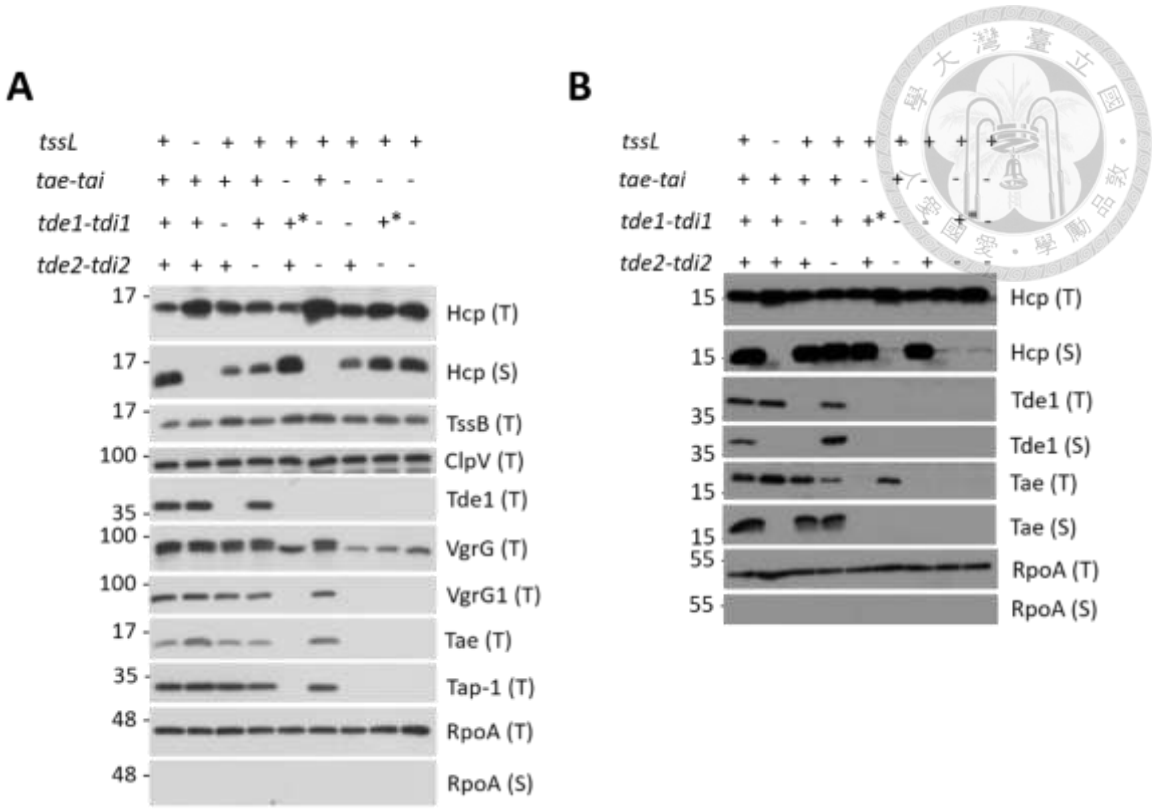

Figure 15. Effects of toxin-immunity gene pairs in Hcp secretion are different when grown in I-medium and LB medium. Secretion assay of *A. tumefaciens* wild type strain C58 (positive control), various single, double, and triple toxin-immunity gene pair mutants, and Δ*tssL* (negative control). Total (T) and secreted (S) fractions were collected from *A. tumefaciens* strains grown in in I medium (pH 5.5) (A) or LB (pH 7.0) (B) for western blotting analysis in detecting indicated proteins with specific antibodies. Both VgrG1 (upper band) and VgrG2 (lower band) can be detected by α-VgrG antibody against purified VgrG1-His proteins while VgrG1 but not VgrG2 can be recognized by α-VgrG1 antibody against synthetic VgrG1 C-terminus (Bondage et al., 2016). Asterisk:

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tae-tai deletion has polar effects in downstream protein Tde1 accumulation (see details in text). Non-secreted RpoA protein serves as an internal control. Molecular weight markers (in kDa) were indicated on the left.

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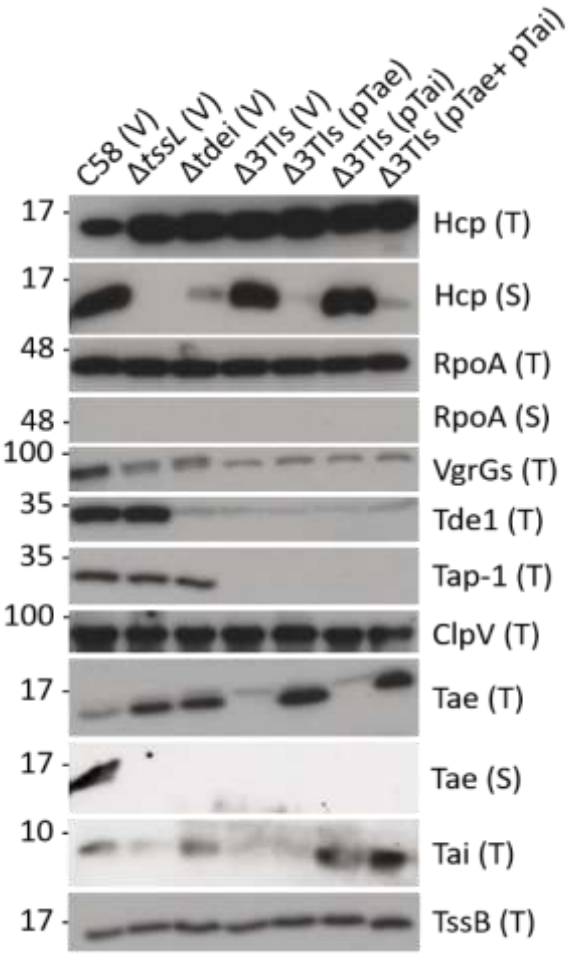

Figure 16. Effect of *tae* in Hcp and VgrG1/2 secretion in Δ3TIs mutant. Secretion assay of *A. tumefaciens* wild type C58, Δ*tssL,* Δ*tdei,* and Δ3TIs harboring vector (V) only or complementation plasmid(s). Total (T) and secreted (S) fractions were collected from *A. tumefaciens* strains grown in I medium (pH 5.5) for western blot analysis using various antibodies as indicated. pTae: pRL662 expressing Tae, pTai: pTrc200 expressing Tai. Molecular weight markers (in kDa) were indicated on the left.

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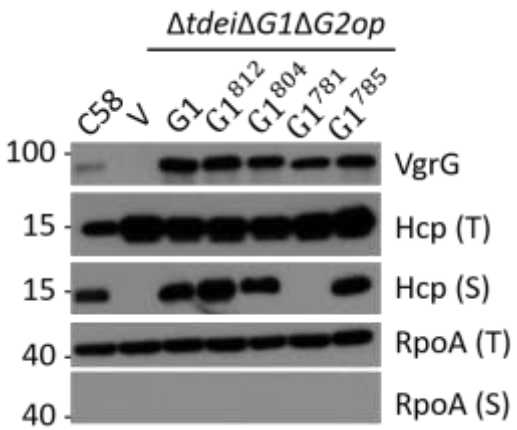

Figure 17. Effect of full length and various truncated VgrG1 proteins on Hcp secretion in Δ*tdei*Δ*vgrG1*Δ*vgrG2* mutant (Δ*tdei*Δ*G1*Δ*G2*). Secretion assay of *A. tumefaciens* wild type C58 or Δ*tdei*Δ*G1*Δ*G2* mutant strain harboring pRL662 (V) or its derivative overexpressing full-length and truncated VgrG1 proteins. Total (T) and secreted (S) fractions were collected from *A. tumefaciens* strains grown in I medium (pH 5.5) for western blot analysis using various antibodies as indicated. Molecular weight markers (in kDa) were indicated on the left.

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TABLES

Table 1. Bacteriophage proteins homologous to T6SS components.

T6SS protein	Bacteriophage protein*	Subcomplex**	Reference(s)
Hcp	gp19	Tube	(Leiman et al., 2009)
VgrG	gp27/gp5	Spike	(Leiman et al., 2009)
PAAR	gp5.4	Tip	(Shneider et al., 2013)
TssBC	gp18	Sheath	(Leiman et al., 2009; Lossi et al., 2013; Kudryashev et al., 2015)
P. aeruginosa TssA1	gp6	Inner baseplate	(Planamente et al., 2016)
TssE	gp25	Inner baseplate	(Leiman et al., 2009; Lossi et al., 2011; Taylor et al., 2016)
TssF	gp6	Inner baseplate	(Brunet et al., 2015; Planamente et al., 2016; Taylor et al., 2016)
Uropathogenic *E. coli* TssG	gp7	Inner baseplate/ intermediate baseplate	(Taylor et al., 2016)

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EAEC TssG	gp53	Inner baseplate	(Brunet et al., 2015)
V. cholerae TssG	gp7/gp53	Inner baseplate/ intermediate baseplate	(Nazarov et al., 2017)
P. aeruginosa TssK	gp8	Intermediate baseplate	(Planamente et al., 2016)
EAEC TssK	Siphophage receptor binding protein	Receptor binding protein	(Nguyen et al., 2017b)
V. cholerae TssK	gp10	Tail fiber network	(Nazarov et al., 2017)

*: Homologous proteins in T4 phage unless there is indication.

**: The nomenclature follows this reference (Taylor et al., 2016).

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Table 2. Reported interaction relationship of T6SS baseplate proteins with other T6SS components interaction network.

Organism	Proteins	Interaction	Method(s) used	Reference(s)
EAEC	TssA-TssA	+	B2H; size exclusive chromatography (SEC)	(Zoued et al., 2016)
P. aeruginosa	TssA-TssA	+	B2H; *in vitro* crossing linking assay; analytical ultracentrifugation (AUC)	(Planamente et al., 2016)
A. tumefaceins	TssA-TssA	+	B2H	This study
EAEC	TssA-TssE	+	B2H; surface plasmon resonance	(Zoued et al., 2016)
P. aeruginosa	TssA-TssE	-	B2H	(Planamente et al., 2016)
EAEC	TssA- TssF	-	B2H	(Brunet et al., 2015; Zoued et al., 2016)
P. aeruginosa	TssA-TssF	+	Pull-down assay combining with MS analysis; B2H	(Planamente et al., 2016)
EAEC	TssA-TssG	-	B2H	(Brunet et al., 2015; Zoued et al., 2016)

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P. aeruginosa	TssA-TssG	-	B2H	(Planamente et al., 2016)
EAEC	TssA-TssK	+	B2H; co-IP with cell lysate of W3110 producing the proteins	(Zoued et al., 2013; Zoued et al., 2016)
P. aeruginosa	TssA-TssK	+	Pull-down assay combining with MS analysis; B2H	(Planamente et al., 2016)
A. tumefaciens	TssA-TssK	+	Co-purification assay in *A. tumefaceins*	This study
EAEC	TssA-TssB	-	B2H	(Zoued et al., 2016)
P. aeruginosa	TssA-TssB	+	Pull-down assay combining with MS analysis; co-purification in *E. coli* combining with SEC	(Planamente et al., 2016)
EAEC	TssA-TssC	+	B2H; surface plasmon resonance	(Zoued et al., 2016)
P. aeruginosa	TssA-ClpV	+	Pull-down assay combining with MS analysis; B2H	(Planamente et al., 2016)
EAEC	TssA-Hcp	+	B2H; surface plasmon resonance	(Zoued et al., 2016)
P. aeruginosa	TssA-Hcp	+	Pull-down assay combining with MS analysis; B2H	(Planamente et al., 2016)

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EAEC	TssA-VgrG	+	B2H	(Zoued et al., 2016)
P. aeruginosa	TssA-VgrG	+	B2H	(Planamente et al., 2016)
A. tumefaciens	TssA-VgrG	+	Co-purification in *E. coli* and in *A. tumefaciens*	This study
EAEC	TssA-TssJLM complex	+	EAEC affinity column purification with strep-tagged TssJ	(Zoued et al., 2016)
EAEC	TssE-TssF	+	Co-IP with cell lysate of W3110 producing the proteins	(Brunet et al., 2015)
EAEC	TssE-TssG	+	B2H; Co-IP with cell lysate of W3110 producing the proteins	(Brunet et al., 2015)
EAEC	TssE-TssK	-	B2H	(Zoued et al., 2013)
EAEC	TssE-VgrG	+	reconstitution experiment mixing cleared cell lysate of cells producing VSV-G-tagged VgrG, TssF, TssG and TssE than IP with anti-VSV-G beads	(Brunet et al., 2015)
A. tumefaceins	TssE-VgrG	-	Co-purification in *E. coli* and in *A. tumefaciens*	This study
EAEC	TssF-TssF	-	B2H	(Brunet et al., 2015)
A. tumefaciens	TssF-TssF	+	B2H	This study

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EAEC	TssF-TssG	+	B2H; co-IP using cell lysate of W3110 producing the proteins	(Brunet et al., 2015)
EAEC	TssF-TssK	+	B2H with co-expression of TssG in the host cell; co-IP using cell lysate of W3110 producing the proteins ※Co-express with TssF	(Brunet et al., 2015; Nguyen et al., 2017)
S. marcescens	TssF-TssK	+	Pull-down assay; *in vitro* SEC	(English et al., 2014)
EAEC	TssF-TssB	-	B2H	(Brunet et al., 2015)
EAEC	TssF-TssC	-	B2H	(Brunet et al., 2015)
EAEC	TssF-Hcp	+	B2H and co-IP	(Brunet et al., 2015)
EAEC	TssF-VgrG	+	B2H with co-expression of TssG in the host cell; reconstitution experiment mixing cleared cell lysate pf cells producing VSV-G-tagged VgrG, TssF, TssG and TssE then IP with anti-VSV-G beads	(Brunet et al., 2015)
A. tumefaceins	TssF-VgrG	+	Co-purification in *E. coli*	This study
EAEC	TssF-TssJLM	-	B2H	(Brunet et al., 2015)
A. tumefaciens	TssG-TssG	+	B2H	This study
EAEC	TssG-TssK	+	B2H; co-IP using cell lysate of W3110 producing the proteins	(Nguyen et al., 2017)

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			※Co-express with TssF	
S. marcescens	TssG-TssK	+	Pull-down assay; *in vitro* SEC	(English et al., 2014)
A. tumefaceins	TssG-TssK	+	B2H	This study
EAEC	TssG-TssB	-	B2H	(Brunet et al., 2015)
EAEC	TssG-TssC	+	B2H	(Brunet et al., 2015)
EAEC	TssG-VgrG	+	B2H with co-expression of TssF in the host cell	(Brunet et al., 2015)
EAEC	TssG-VgrG	+	Reconstitution experiment mixing cleared cell lysate pf cells producing VSV-G-tagged VgrG, TssF, TssG and TssE than IP with anti-VSV-G beads	(Brunet et al., 2015)
A. tumefaciens	TssG-VgrG	+	Co-purification in *E. coli*	This study
EAEC	TssG-TssJL	-	B2H	(Brunet et al., 2015)
EAEC	TssG-TssM	+	B2H; co-IP with cell lysate of W3110 producing the proteins	(Brunet et al., 2015)
EAEC	TssK-TssK	+	B2H; co-IP with cell lysate of W3110 producing the proteins; SEC	(Zoued et al., 2013; Nguyen et al., 2017)
S. marcescens	TssK-TssK	+	SEC; AUC	(English et al., 2014)

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A. tumefaciens	TssK-TssK	+	B2H	This study
EAEC	TssK-TssB	-	B2H	(Zoued et al., 2013)
EAEC	TssK-TssC	+	B2H; Co-IP with cell lysate of W3110 producing the proteins	(Zoued et al., 2013)
EAEC	TssK-Hcp	+	B2H; Co-IP with cell lysate of W3110 producing the proteins	(Zoued et al., 2013)
EAEC	TssK-VgrG	-	B2H	(Zoued et al., 2013)
A. tumefaciens	TssK-VgrG	+	Co-purification in *E. coli* and in *A. tumefaceins*	This study
EAEC	TssK-TssJ	-	B2H	(Zoued et al., 2013)
EAEC	TssK-TssLM	+	B2H; co-IP with cell lysate of W3110 producing the proteins	(Zoued et al., 2013; Nguyen et al., 2017)
A. tumefaciens	VgrG-TssBC	+	Co-purification in *E. coli*	(Lin et al., 2013)
A. tumefaciens	VgrG-Hcp	+	Co-purification in *E. coli*; co-IP	(Lin et al., 2013)
V. cholerae	VgrG-VgrG	+	Pull-down assay	(Pukatzki et al., 2007)

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Table 3. Bacterial strains and plasmids used in this study.

Strains	EML No.	Relevant characteristics	Sources
A. tumefaiens			
C58	EML530	Wild type virulent strain	Eugene Nester
Δ*G1*	EML1134	*vgrG1* deletion mutant	(Lin et al., 2013)
Δ*tde1*	EML1142	*tde1* deletion mutant	(Ma et al., 2014)
Δ*tssL*	EML1073	*tssL* deletion mutant, used as negative control of T6SS secretion	(Ma et al., 2009)
Δ*tde1-tdi1*	EML3392	*tde1-tdi1* effector-immunity deletion mutant	(Ma et al., 2014)
Δ*tde2-tdi2*	EML3551	*tde2-tdi2* effector-immunity deletion mutant	(Ma et al., 2014)
Δ*tae-tai*	EML3553	*tae-tai* effector immunity deletion mutant	(Lin et al., 2013)
Δ*tdei*	EML3559	Double deletion mutant of *tde1-tdi1* and *tde2-tdi2* effector-immunity pair	(Ma et al., 2014)
Δ*tae-tai*Δ*tde1-tdi1*	EML3555	Double deletion mutant of *tde1-tdi1* and *tae-tai* effector-immunity pair	Lay-Sun Ma
Δ*tde2-tdi2*Δ*tae-tai*	EML3557	Double deletion mutant of *tae-tai* and *tde2-tdi2* effector-immunity pair	Lay-Sun Ma
Δ3TIs	EML3561	Triple deletion mutant of *tae-tai*, *tde1-tdi1* and *tde2-tdi2* effector-immunity pairs	(Ma et al., 2014)

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Δ*tap-1*	EML4290	Deletion mutant of Tde1 adaptor/chaperone gene *tap-1*	(Ma et al., 2014)
Δ*atu3641*	EML3406	Deletion mutant of Tde2 adaptor chaperone gene *atu3641*	(Bondage et al., 2016)
Δ*G1*Δ*G2*	EML1289	*vgrG* double deletion mutant	(Lin et al., 2013)
Δ*tssK*	EML1078	*tssK* deletion mutant	(Lin et al., 2013)
Δ*tdei*Δ*G1*	EML5123	*tde-tdi* double deletion mutant with additional deletion of *vgrG1*	Devanand Bondage
Δ*tdei*Δ*G1*Δ*G2 op*	EML5130	*tde-tdi* double deletion mutant with additional deletion of *vgrG1* and *vgrG2* operon	This study
Δ*tdei1*Δ*tssK*	EML5141	*tde1-tdi1* deletion mutant with additional deletion of *tssK*	This study
Δ*tdei2*Δ*tssK*	EML5138	*tde2-tdi2* deletion mutant with additional deletion of *tssK*	This study
Δ*tdei*Δ*tssK*	EML5136	*tde-tdi* double deletion mutant with additional deletion of *tssK*	This study
E. coli			
DHM1	EML5135	Host for B2H assay	(Karimova et al., 2005)
BL21(DE3)	EML117	Host for gene overexpression driven by T7 promoter, used as host for co-purification assay	(Studier et al., 1990)
Plasmids			
pT18	EML3589	Empty vector containing C-terminus-fused adenylate cyclase T18 domain for B2H assay, Ap^R	(Karimova et al., 1998)

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pT25	EML3590	Empty vector containing N-terminus-fused adenylate cyclase T25 domain for B2H assay, Cm^R	(Karimova et al., 1998)
pT18-TssA	EML2752	TssA was cloned into pT18, Ap^R	Jer-Sheng Lin
pT25-TssA	EML2750	TssA was cloned into pT25, Cm^R	Jer-Sheng Lin
pT18-TssE	EML2748	TssE was cloned into pT18, Ap^R	Jer-Sheng Lin
pT25-TssE	EML2736	TssE was cloned into pT25, Cm^R	Jer-Sheng Lin
pT18-TssF	EML2747	TssF was cloned into pT18, Ap^R	Jer-Sheng Lin
pT25-TssF	EML2735	TssF was cloned into pT25, Cm^R	Jer-Sheng Lin
pT18-TssG	EML2746	TssG was cloned into pT18, Ap^R	Jer-Sheng Lin
pT25-TssG	EML2734	TssG was cloned into pT25, Cm^R	Jer-Sheng Lin
pT18-TssK	EML3334	TssK was cloned into pT18, Ap^R	Lay-Sun Ma
pT25-TssK	EML3330	TssK was cloned into pT25, Cm^R	Lay-Sun Ma
pT18-VgrG1	EML3336	VgrG1 was cloned into pT18, Ap^R	Lay-Sun Ma
pT18-ZIP and pT25-ZIP	EML3591	Positive control for B2H assay, Ap^R and Cm^R	(Karimova et al., 1998)
pRL662	EML315	broad host range expression vector derived from pBBR1MCS-2, Gm^R	(Vergunst et al., 2000)
pTrc200	EML904	*pVS1* origin *lacI*^{q}, *trc* promoter expression vector, Sp^R	(Schmidt-Eisenlohr et al., 1999)
pTde1-Tdi1	EML4277	*tde1* and tdi1 were cloned into pTrc200, Sp^R	Lay-Sun Ma
pTde2HADA	EML4797	*tde2* catalytic site mutant was cloned into pRL663, Gm^R	Devanand Bondage

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pTap-1	EML4255	*tap-1* was cloned into pTrc200, Sp^R	(Ma et al., 2014)
p3641	EML4785	*atu3641* was cloned into pTrc200, Sp^R	(Bondage et al., 2016)
pET28a(+)	EML2485	Overexpression vector to generate N-terminal His-tagged protein driven by T7 promoter, Km^R	Novagen
pET22b(+)	EML188	Overexpression vector to generate N-terminal His-tagged protein driven by T7 promoter, Ap^R	Novagen
pET28a-VgrG1	EML4800	*vgrG1* was cloned into pET28a for N-terminal His tag, Km^R	Devanand Bondage
pET22b-VgrG1	EML1836	*vgrG1* was cloned into pET22b for C-terminal His tag, Ap^R	(Lin et al., 2013)
pTrc-TssA	EML4001	*tssA* was cloned into pTrc200, Sp^R	Jer-Sheng Lin
pTrc-TssE	EML4042	*tssE* was cloned into pTrc200, Sp^R	Jer-Sheng Lin
pTrc-TssK	EML5115	*tssK* was cloned into pTrc200, Sp^R	This study
pRL-TssF-HA	EML1882	*tssF* with C-terminal HA tag was cloned into pRL662, Gm^R	Jer-Sheng Lin
pRL-TssG-HA	EML1881	*tssG* with C-terminal HA tag was cloned into pRL662, Gm^R	Jer-Sheng Lin
pRL-VgrG1-His	EML2050	*vgrG1* with C-terminal His tag was cloned into pRL662, Gm^R	Jer-Sheng Lin
pRL-VgrG2-His	EML2051	*vgrG2* with C-terminal His tag was cloned into pRL662, Gm^R	Jer-Sheng Lin
pRL-TssK-His	EML2040	*tssK* with C-terminal His tag was cloned into pRL662, Gm^R	Jer-Sheng Lin

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pRL-TssK	EML1301	*tssK* was cloned into pRL662, Gm^R	(Lin et al., 2013)
pTrc-Tap-1-Tde1-Tdi1-PAAR	EML4275	*tap-1, tde1, tdi1* and *PAAR* are cloned into pTrc200, Sp^R	(Ma et al., 2014)
pTae	EML1616	*tae* was cloned into pRL662, Gm^R	(Lin et al., 2013)
pTai	EML4228	*tai* was cloned into pTrc200, Sp^R	(Ma et al., 2014)
pRL-VgrG1	EML1422	*vgrG1* was cloned into pRL662, Gm^R	(Lin et al., 2013)
$pG1^{812}$	EML4567	*vgrG1* with amino acid residue 813 to 816 deleted was cloned into pRL662, Gm^R	(Bondage et al., 2016)
$pG1^{804}$	EML4568	*vgrG1* with amino acid residue 805 to 816 deleted was cloned into pRL662, Gm^R	(Bondage et al., 2016)
$pG1^{785}$	EML4599	*vgrG1* with amino acid residue 786 to 816 deleted was cloned into pRL662, Gm^R	(Bondage et al., 2016)
$pG1^{781}$	EML4598	*vgrG1* with amino acid residue 782 to 816 deleted was cloned into pRL662, Gm^R	(Bondage et al., 2016)
pJQ200KS-*vgrG1*	EML954	*vgrG1*-flanking sequences was cloned into pJQ200KS plasmid to generate in-frame deletion mutant, Gm^R	(Lin et al., 2013)
pJQ200KS-*vgrG2* operon	EML2689	*vgrG2* operon-flanking sequences was cloned into pJQ200KS plasmid to generate in-frame deletion mutant, Gm^R	Jer-Sheng Lin

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pJQ200KS-*tssK*	EML941	*tssK*-flanking sequences was cloned into pJQ200KS plasmid to generate in-frame deletion mutant, Gm^R	(Lin et al., 2013)

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Table 4. Primers used in this study. Restriction enzyme sites are shown with underline.

Name	Sequence (5' to 3')	Related construct(s)
*tssK*_BamHI_F	AGTGGATCCCCGCGTCCGCACGGGAG	pTrc-TssK
*tssK*_SalI_R	AAGTCGACTCATTCGCGTAACGCCCACATTTCC	pTrc-TssK
dG1_F	ATGCGCGTTAACTTTGACAC	Δ*tdei*Δ*vgrG1*
dG1_R	ATTATGGGTGTGTCGTTCAT	Δ*tdei*Δ*vgrG1*
G2op_F	GAACAGCCTGACAATCCTGT	Δ*tdei*Δ*vgrG1*Δ*vgrG2* operon
G2op_R	CAGTGCCTGATAGACGTTGT	Δ*tdei*Δ*vgrG1*Δ*vgrG2* operon
dtssK_F	ATGAAGCTTGCACTCAAGAA	Δ*tdei1*Δ*tssK*, Δ*tdei2*Δ*tssK*, Δ*tdei*Δ*tssK*
dtssK_R	GAGGGGTTGTCCGTGCTC	Δ*tdei1*Δ*tssK*, Δ*tdei2*Δ*tssK*, Δ*tdei*Δ*tssK*

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