A glycine zipper motif governs translocation of type VI secretion toxic effectors across the cytoplasmic membrane of target cells

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ABSTRACT

Type VI secretion systems (T6SSs) can deliver diverse toxic effectors into eukaryotic and bacterial cells. Although much is known about the regulation and assembly of T6SS, the translocation mechanism of effectors into the periplasm and/or cytoplasm of target cells remains elusive. Here we used the Agrobacterium tumefaciens DNase effector Tde1 to unravel the mechanism of translocation from attacker to prey. We demonstrate that Tde1 loading onto the secretion machinery is mediated via binding to its adaptor through the N-terminus, which harbours continuous copies of GxxxG motifs resembling the glycine zipper structure found in proteins involved in the membrane channel formation. By amino acid substitutions on a conserved glycine zipper motif, we showed G^{38}xxxG^{43} motif governs the delivery into target cells by permeabilizing the cytoplasmic membrane. The findings demonstrate effector itself mediates target cell entry. Considering the widespread presence of GxxxG motifs in bacterial effectors and pore-forming toxins, we proposed that glycine zipper mediated permeabilization is a conserved mechanism used by bacterial effectors for translocation across target cell membranes.

Key words: Type VI secretion system, DNase effector, antibacterial, glycine zipper, translocation, Agrobacterium tumefaciens

INTRODUCTION

In a complex microbial community, bacteria have evolved versatile secretion systems for export or import of substrates across their membranes in response to different environmental cues. Each of specialized protein secretion systems (type I to X secretion system [TISS to TXSS]) \textsuperscript{1,2,3} can recognize specific substrates for secretion and translocation across one or multiple membranes. The type VI secretion system (T6SS) is a molecular weapon deployed by many Proteobacteria for pathogenesis, antagonism, or nutrient acquisition \textsuperscript{4}. The T6SS effectors discovered so far exert functions in antibacterial, anti-eukaryotic, and metal acquisition \textsuperscript{5-8}. The most established T6SS effectors are
bacterial toxins, in which bacteria also produce cognate immunity proteins to prevent self-intoxication and toxicity in the sibling cells.

T6SS is a multiprotein complex, composed of at least 13 conserved core proteins resembling a phage tail structure, that extends from the cytoplasm to the outer membrane of the attacker cell\(^9,10\). The T6SS machine consists of the Tss(J)LM membrane complex (MC), Tss(A)EFGK base plate (BP), TssBC contractile sheath, and Hcp-VgrG-PAAR puncturing device. The MC interacts with the BP\(^{11,12}\), which serves as a docking site of VgrG-PAAR effector complex to initiate polymerization of the tail\(^13\). The tail is composed of the Hcp inner tube and TssBC outer sheath, and when triggered, the sheath contracts and ejects out the effector decorated puncturing device into extracellular milieu or target cells\(^14,15\).

The T6SS has multiple strategies for delivering diverse effectors. On the basis of the known effectors and their transport mechanisms, effectors can be classified as “specialized” or “cargo” effectors\(^9,16\). Specialized effectors are fused to either of the C-termini of three core structural proteins (Hcp, VgrG, or PAAR) while cargo effectors interact directly or require a specific chaperone/adaptor to be loaded into the lumen of the Hcp tube or onto the VgrG spike prior to secretion. Antibacterial effectors can act in the cytoplasm, membrane, or periplasm of the target cells\(^5,6,8\). It is not known how effectors that target the cytoplasm breach both the outer and inner membranes of target cells.

Glycine zipper structure consisting of repetitive GxxxG motifs is commonly found in membrane-associated proteins\(^17\) and bacterial toxins\(^18,19\). Glycine zipper motifs are known be involved in toxicity of some bacterial effectors for membrane channel formation. For examples, the transmembrane domain (TMD) of a vacuolating toxin, VacA of *Helicobacter pylori* encodes three GxxxG motifs forming helix-helix packing interactions\(^19\), which are required for the vacuolation and membrane channelling contributing to VacA toxicity\(^20\). Type I secretion effectors CdzC and CdzD of *Caulobacter crescentus* and T6SS effector Tse4 of *Pseudomonas aeruginosa*, also possess glycine zipper motifs involved in antibacterial activity\(^21,22\). Expression of Tse4 disrupted the proton motive force of the inner membrane while CdzC and CdzD form surface aggregation for contact-
dependent killing of target cells. However, how glycine zipper motifs of Tse4 and CdzCD involved in toxicity remain unknown.

A T6SS-encoding locus is highly conserved in the genome of plant pathogenic bacterium Agrobacterium tumefaciens and the apparatus functions as an antibacterial weapon. We previously revealed that A. tumefaciens strain C58 deploys two Type VI DNase effectors (Tde1 and Tde2) as the major antibacterial weapons, in which the cognate immunity proteins (namely Tdi1 and Tdi2) prevent autointoxication. Both Tde1 and Tde2 harbour a novel C-terminal Ntox15 toxin domain containing an HxxD catalytic motif required for its DNase activity. Tde1 requires its cognate chaperone/adaptor Tap1 for loading onto VgrG1 for secretion.

By obtaining an uncoupling Tde1 variant that remains capable for binding to Tap1 for export but deficient in membrane permeability, we revealed the secretion and translocation mechanism of Tde1 from the attacker cell to the target cell. We showed that the N-terminal region of Tde1 harbouring repetitive glycine zipper motifs is sufficient for interacting with Tap1 for secretion. Once secreted, a conserved glycine zipper motif is necessary for translocation across target cell membranes. The findings demonstrate a new role of glycine zipper motif(s) in effector delivery into target cells.

RESULTS

Tde1 can cause DNase-independent growth inhibition of prey cells

Our previous study showed that overexpression of Tde1 in Agrobacterium tumefaciens C58 caused growth inhibition, and the immunity protein Tdi1 only partially protected against this cytotoxicity. We hypothesized that Tde1 has domains apart from the DNase domain that contribute to its toxicity. In addition to the C-terminal Ntox15 DNase domain (amino acid 99-247), Tde1 has a predicted transmembrane domain (TMD, 22-42) (Fig.1A). Thus, three fragments of Tde1, that being the N-terminal, N-Tde1(1-97) and two C-terminal regions, C1-Tde1(49-278), and C2-Tde1(98-278) were tested for toxicity. To avoid confounding effects by the DNase activity, substitutions of catalytic residues (H190A, D193A) were introduced in the C1-Tde1 and the full length wide-type Tde1 to
become C1-Tde1(M) and Tde1(M) respectively (Fig.1A). Ectopic expression in *Escherichia coli* (DH10B) under an IPTG inducible promoter showed that N-Tde1 was sufficient to inhibit growth (Fig.1B). Tde1(M), but not the C1-Tde1(M), is growth inhibitory. Surprisingly, C2-Tde1(WT), which retains the wild type DNase catalytic residues, was not sufficient to inhibit growth. Evidence suggests the N-terminal region of Tde1 is sufficient to confer toxicity under conditions tested and that the C-terminal DNase domain requires the N-terminus for it to cause toxicity.

To test Tdi1, the immunity protein for the DNase toxicity of Tde1, can also neutralize the N-Tde1 toxicity, the Tde1 variants were co-expressed with the Tdi1. The result showed that Tdi1 could not rescue the growth inhibition caused by the N-Tde1 and Tde1(M) (Fig 1C). This indicates that Tdi1 cannot neutralize the N-terminus-mediated toxicity.

A glycine zipper motif in N-terminus of Tde1 is required for toxicity and enhanced membrane permeability

To get an insight into the cause of growth inhibition by N-terminus of Tde1, we used N-Tde1 region as a query to search against the NCBI non redundant (nr) database and identified Tde1 homologues encoded in the T6SS gene clusters of *Agrobacterium/Rhizobium* as well as tape measure proteins (TMP) encoded in genomes of *Paraburkholderia/Burkholderia* (Fig 1D, Fig S1). We noticed the striking conservation of continuous copies of GxxxG motifs (12-51), which resembles the glycine zipper motifs overrepresented in membrane proteins and reported to be involved in the membrane channel formation. Thus, we hypothesized that these repetitive glycine zipper motifs are necessary for membrane permeability and are the cause for N-Tde1 toxicity.

To test this, two highly conserved glycine residues at position 39 and 43 of a glycine zipper motif were substituted with leucine (G39L and G43L), and the resulting N-Tde1 and Tde1(M) variants were named as N-Tde1GLGL, Tde1(M)GLGL respectively. The growth analysis of *E. coli* DH10B cells showed that both N-Tde1GLGL and Tde1(M)GLGL lost the ability to cause growth inhibition (Fig.2A). Similar results were also observed when they were overexpressed in *A. tumefaciens* Δtde1 mutant (Fig. S2), indicating that the G39xxxG43 glycine zipper motif of Tde1 is responsible for the observed toxicity.
Next, we tested whether N-Tde1 can increase *E. coli* inner membrane permeability. To do so, we used the β-galactosidase activity assay to measure the entry of ortho nitrophenyl galactopyranoside (ONPG) (301 Da) into the cytosol. ONPG normally requires a functional permease LacY to enter into the cytosol but can enter if the inner membrane is permeabilized/compromised. The N-Tde1 and Tde1(M) as well as their glycine zipper substitution variants were expressed in *E. coli* BW25113ΔlacY carrying β-galactosidase (pYTA-lacZ). The BW25113ΔlacY(pYTA-lacZ) complemented with lacY was used as a positive control.

The results showed that cells expressing either N-Tde1 or Tde1(M) had similar β-galactosidase activity as LacY expressing cells. In contrast, cells expressing N-Tde1GLGL and Tde1(M)GLGL only exhibited background level activity as the negative controls (Fig 2B). These results indicate that the N-Tde1 and Tde1(M) are able to increase membrane permeability depending on the G39xxxG43 motif. The data also suggest that the N-terminus-mediated growth inhibition is caused by its ability to enhance inner membrane permeability through glycine zipper motifs.

To further analyse the extent of enhanced membrane permeabilization, cells from the same experiment were normalized to the same OD600 and stained with Hoechst and propidium iodide (PI). Hoechst (616 Da) is a nucleic acid staining dye which is permeable to live Gram-negative bacterial cells while PI (668.4 Da) can enter through a compromised inner membrane or dead cells. The PI/Hoechst staining showed strong PI signals in cells expressing N-Tde1 and Tde1 (M) but no or weak signals were detected in cells expressing N-Tde1GLGL, Tde1(M)GLGL, or vector control, indicating that N-Tde1 is able to enhance membrane permeability to allow molecules with size 668.4 Da to pass (Fig 2C). Hoechst signals were detected in all cells but stronger in cells expressing N-Tde1 or Tde1(M).

The cells used for membrane permeability assays were also collected for counting viable cells. Accordingly, there was no obvious difference on the number of viable cells among the strains tested (Fig 2D), suggesting that the detected enhanced PI fluorescence in cells expressing N-Tde1 and Tde1(M) is due to compromised membrane permeabilization but not cell death.
We next determined whether the N-terminus of Tde1 is bacteriostatic or bacteriolytic. E. coli cells were induced with IPTG to express N-Tde1 or Tde1(M) and after one hr, washed with fresh media without IPTG for continuous cultivation. We found that growth was recovered when cells were washed of the IPTG inducer, in contrast to the growth inhibition of cells with continuous IPTG induction (Fig 2E). The combined data suggest that the N-terminus of Tde1 is sufficient to facilitate membrane permeability for bacteriostatic toxicity, and such activity requires the conserved G$^{39}$xxxG$^{43}$ glycine zipper motif.

**The N-terminus of Tde1 is necessary and sufficient for Tap1 interaction**

Tap1 is the adaptor for loading Tde1 onto VgrG$^{1,23,28}$. However, the mechanism that Tde1 and Tap1 interact remains undefined. We first performed co-immunoprecipitation (co-IP) assay to identify the specific region of Tde1 that can interact with Tap1 in A. tumefaciens. The HA-tagged Tde1 variants were expressed in $\Delta$tde1 and anti-HA agarose bead was used to co-precipitate the interacting proteins followed by western blotting to detect Tde1 variants and Tap1. The results showed that the N-Tde1 and Tde1(M) interact with Tap1 but not the C-terminal variants, C1-Tde1(M) and C2-Tde1 (Fig.3A). N-Tde1$^{GLGL}$ and Tde1(M)$^{GLGL}$ remain capable of interacting with Tap1 (Fig.3A).

Next, we determined whether the Tap1 can interact with Tde1 in E. coli where T6SS is absent. Similar to the results in A. tumefaciens, the N-Tde1 and Tde1(M) interacted with Tap1 but not the C-terminal variants (Fig. 3B). Tap1 interaction with N-Tde1$^{GLGL}$ was also detected, but since Tde1(M)$^{GLGL}$ was unstable in E. coli, intact Tde1$^{GLGL}$ was barely detected and the interaction with Tap1 was similarly diminished (Fig. 3B). The co-IP results in A. tumefaciens and E. coli together suggest that Tap1 interacts with Tde1 through the N-terminus and that the G39L and G43L substitution does not affect Tde1-Tap1 interaction. However, G$^{39}$xxxG$^{43}$ motif is crucial to maintain integrity of Tde1(M) when expressed in E. coli (Fig. 3B).

**The N-terminus of Tde1 is necessary and sufficient for secretion**

Because N-Tde1 interacts with Tap1, we hypothesized that this region is required for Tde1 secretion. Thus, we performed secretion assay by expressing the various HA-tagged Tde1 variants in $\Delta$tde1, a deletion mutant lacking both tde1-tdi1 and tde2-tdi2 toxin
immunity pairs. Both cellular and extracellular fractions were collected to determine their expression and secretion, respectively. The results showed that indeed all Tde1 variants containing N-terminus is secreted but not the C-terminus, C1-Tde1(M). The secretion was in a T6SS-dependent manner as secretion was abrogated in ΔtdeiΔtssK, which lacks both tdei and tssk encoding the baseplate component. N-Tde1GLGL and Tde1(M)GLGL are also stably expressed and secreted (Fig. 3C). The data suggest that N-terminus of Tde1 is necessary and sufficient for secretion and that the G39L and G43L substitution did not interfere with the secretion capacity of Tde1. Accordingly, Hcp secretion levels are highly correlated with Tap1-Tde1 interaction and secretion of Tde1 variants (Fig. 3C). The data also confirmed the requirement of the Tap1-Tde1 interaction for Tde1 secretion and supported our previous finding that Tde loading onto VgrG is critical for active T6SS secretion.

G39xxxG43 motif of Tde1 is required for target cell delivery

Because the G39xxxG43 glycine zipper motif located in N-Tde1 increased the membrane permeability but was not required for secretion, we hypothesized that G39xxxG43 is responsible for inserting Tde1 into the inner membrane and delivering it into the cytoplasm of target cells. Here, we engineered each of Tde1 variants fused to superfolder green fluorescence protein (sfGFP) with a flexible (GGGS) linker between Tde1 and sfGFP to avoid the Tde1 functional/structural interference by GFP. The sfGFP fused Tde1 variants were expressed in A. tumefaciens Δtdei and ΔtdeiΔtssK mutants. The secretion assay results showed that N-Tde1-sfGFP and Tde1(M)-sfGFP but not C1-Tde1(M)-sfGFP are secreted and the G39L and G43L substitution did not compromise the secretion (Fig 4A). No proteins were observed in the extracellular fractions of Δtdei/Δtssk mutants, demonstrating that T6SS-dependent secretion.

To investigate the translocation of Tde1 variants, we mixed A. tumefaciens Δtdei, expressing sfGFP-fused Tde1 variants, with E. coli cells expressing mCherry. A. tumefaciens expressing sfGFP only (Vector-sfGFP) was used as a negative control. After co-culture, we imaged populations for mCherry (false coloured in blue) and GFP (green) to detect E. coli and A. tumefaciens, respectively. We merged images to identify cyan colored cells (overlayed blue and green signals), which represent E. coli cells with
translocated Tde1 variants carrying sfGFP. We were able to observe cyan fluorescence when A. tumefaciens expressing N-Tde1-sfGFP and Tde1(M)-sfGFP was co-cultured with E. coli mCherry whereas the GFP and mCherry signals were not overlapped in the E. coli cells co-cultured with A. tumefaciens strains expressing GFP only or sfGFP-fused C1-Tde1(M), N-Tde1^GLGL, Tde1(M)^GLGL respectively (Fig. 4B). No cyan fluorescence was observed when N-Tde1-sfGFP and Tde1(M)-sfGFP were expressed in the ∆tdei1∆tssk mutant as the attacker (Fig. S3).

The data suggested that Tde1 is translocated into target cells in a T6SS- and G^39-xxxG^43-dependent manner. Because N-Tde1^GLGL-sfGFP and Tde1(M)^GLGL-sfGFP could be secreted but not translocated into target cells, G^39-xxxG^43 motif is necessary for delivering Tde1 into the target cell.

G^39-xxxG^43 is required for interbacterial competition but dispensable for DNase activity

To assess the role of the G^39-xxxG^43 motif for target cell intoxication in the context of cell-cell contact, we further performed interbacterial competition assays. A. tumefaciens C58 ∆tdei expressing either Tde1-Tdi1 or its glycine zipper variant, Tde1^GLGL-Tdi1, was competed with target E. coli (DH10B) cells. A. tumefaciens ∆tdei (Tde1-Tdei1) exhibited a strong killing activity (~10^3-fold reduction of target cell survival) as compared to the negative controls, the secretion deficient mutants ∆tssL and ∆tdei1∆tssK (Tde1-Tdei1) (Fig 5A). In contrast, no killing activity could be observed for A. tumefaciens ∆tdei expressing Tde1(M)-Tdi1. The killing activity of ∆tdei (Tde1^GLGL-Tdi1) was largely compromised, similar to that of negative controls. We also performed interbacterial competition assays using A. tumefaciens strain 1D1609, which is susceptible to T6SS killing by C58 33. The intra-species killing activity was weaker (~10^2-fold reduced target cell survival), but the killing activity of ∆tdei (Tde1^GLGL-Tdi1) against 1D1609 was not detectable (Fig 5B). The results together indicated that G^39-xxxG^43 motif is required for interbacterial competition at both inter- or intra-species levels. We also performed a secretion assay of these A. tumefaciens attacker strains and as expected, Tde1^GLGL proteins were secreted (Fig. 5C).

It is notable that Tde1^GLGL proteins accumulate at slightly lower levels to that of Tde1 and Tde1(M), which also led to reduced secretion levels of Tde1^GLGL and Hcp.
Although the reduced secretion level may partly contribute to compromised killing effect of \( \Delta tdei(Tde1^{GLGL}-Tdi1) \), the most plausible explanation is that Tde1\(^{GLGL} \) is unable to mediate its translocation across target cell membranes. To exclude the possibility that G39L and G43L substitution may influence its DNase activity, we perform *in vivo* plasmid DNA degradation assay. Tde1, Tde1\(^{GLGL} \), and Tde1(M) were each expressed by tightly controlled arabinose-inducible promoter for *in vivo* plasmid DNA degradation assay in *E. coli* BW25113 as described. As expected, plasmid DNA was completely degraded in cells expressing Tde1, but not in the negative controls, the cells without arabinose induction or cells expressing Tde1(M). As for Tde1\(^{GLGL} \), plasmid DNA was also degraded but not as complete as Tde1 (Fig. 5D). This is not surprising because Tde1(M)\(^{GLGL} \) is prone for degradation in *E. coli* (Fig. 3B). Indeed, no intact Tde1\(^{GLGL} \) could be detected, in contrast to the abundance of Tde1 and Tde1(M) (Fig. 5D). We also generated single G39L and G43L substitution variants (Tde1\(^{G39L} \) and Tde1\(^{G43L} \)) and found that both Tde1\(^{G39L} \) and Tde1\(^{G43L} \) are stably expressed and exhibit wild-type level DNase activity. The degree of plasmid DNA degradation is also correlated with the growth inhibition effect (Fig. 5E and Fig. S2C). The slightly recovery of Tde1\(^{GLGL} \) in growth inhibition is consistent with the instability of Tde1\(^{GLGL} \). The evidence that G39L and G43L substitution of Tde1 abolished inner membrane permeability, translocation, and interbacterial competition but did not affect DNase activity and secretion of Tde1 suggested the G\(^{39}xxxG^{43} \) motif is necessary for delivering Tde1 across the inner membrane into the cytoplasm of target cells.

**DISCUSSION**

Through the dissection of Tde1 DNase effector, we provide strong evidence for a role of the N-terminal glycine-zipper motif(s) of Tde1 in delivering the T6SS effector into target cells. Here, we proposed a model explaining the loading, firing, and translocation of Tde1 (Fig 6). In *A. tumefaciens*, Tde1 DNase activity is neutralized by Tdi1 by binding to C-terminal DNase domain while its N-terminal domain interacts with Tap1 for loading onto VgrG1 (Step 1). The VgrG1-Tap1-Tde1-Tdi1 complex is then recruited onto membrane-associated baseplate, which serves as a docking site for polymerization of Hcp tube and...
Upon TssBC sheath contraction (Step 3), Tap1 and Tdi1 may fall off and Hcp-VgrG-Tde1 puncturing device is then ejected for secretion. In contact with a target cell, Tde1 may be delivered to periplasm of the target cell where Tde1 permeabilises the inner membrane in a G^{39}-xxxG^{43} motif-dependent manner (Step 5). Once delivered, Tde1 exerts its toxicity by attacking DNA for degradation (Step 6).

T6SS cargo effectors often require the specific chaperone/adaptor for loading onto puncturing device for secretion. Our previous findings demonstrated that Tap1, a DUF4123-containing protein, specifically interacts with Tde1 for loading onto VgrG1 for secretion^{23,28}. We now showed that N-terminal region of Tde1 is necessary and sufficient for interaction with Tap1 for secretion and delivery into target cells. The evidence that Tde1^{GLGL} variant remains capable for binding to Tap1 for export but deficient in membrane permeability and translocation demonstrated a distinct role of this G^{39}-xxxG^{43} motif in target cell delivery. Among the nine classes of the Ntox15-containing proteins, the majority of them including Tde1 belong to class I without detectable N-terminal domains (Fig. S1). We identified the presence of glycine zipper motifs overlapping with transmembrane domain (TMD) not only in N-terminal region of all Tde1 orthologs encoded in Rhizobiaceae but also in C-terminal region of tape measure proteins (TMP) encoded in genomes of Paraburkholderia/Burkholderia, likely as a prophage. TMP is a phage protein suggested to have a channel forming activity^{34,35} and as a determinant in connecting host inner membrane proteins for injecting phage genome into bacterial host cells^{36}. Such conservation in Tde1 orthologs suggested that this glycine zipper-mediated delivery could be a common strategy deployed by these bacterial effectors for translocation across target cell membranes. To this end, whether other more distantly related Ntox15-containing proteins or more broadly bacterial toxins harbouring C-terminal toxin domain may also use N-terminal glycine zipper to mediate effector translocation await future investigations. It would be also interesting to investigate whether TMP also employs its C-terminal glycine zipper to mediate phage genome entry into host cells.

Previous study in P. aeruginosa showed that VgrG-loaded Tse6-EgaT6 complex is sufficient to translocate across a lipid bilayer in vitro^{37}, suggesting a role of VgrG-effector complex itself in inserting across the inner membrane of target cells. A recent study further
uncovered a widespread prePAAR motif in N-terminal TMDs of T6SS effectors involved in interaction with Eag family chaperone for export. The findings from the Tap1 and Eag chaperone-mediated T6SS toxins led us to propose that the bacterial toxins harbouring a N-terminal TMD may be protected by its cognate chaperone/adaptor from insertion into membranes in the attacker cell. However, once the effector is secreted in the periplasm of the target cell, specific motifs (such as glycine zippers or perhaps prePAAR) may mediate TMD to insert into the inner membrane for the delivery into the cytoplasm.

A few membrane permeabilizing T6SS toxins have been reported. The *Vibrio cholerae* VasX caused dissipation of membrane potential, leading to membrane permeabilization of target bacterial cells similar to the Tme effectors of *V. parahaemolyticus*, which represents a widespread family of T6SS effectors harbouring C-terminal TMD for membrane disruption. On the other hand, Tse4 disrupts the membrane potential and forms a cation selective pore without membrane permeabilization where the pore cannot even allow the permeability of a relatively smaller molecular weight (ONPG, 300 Da). Distinct from these toxins in which they confer pore forming activity for toxicity, the role of glycine zipper(s) of Tde1 appears to enhance membrane permeability for bringing the toxin domain into target cell cytoplasm.

To date, no structural information is available for Ntox15 superfamily proteins where Tde1 belongs. While N-terminus of Tde1 lacks sequence similarity to any of those known pore-forming toxins, structural modelling showed structural similarity of two helixes containing consecutive glycine zipper motifs to the pore forming domain of pyocin S5. Pyocin S5 can cause ATP leakage and PI permeability potentially to inner membrane after translocation through FptA and TonB1. Tde1 allows the passage of relatively larger molecule, PI (668 Da), suggesting that its N-terminal glycine zipper(s) may form larger pores similar to pyocin S5. G39xxxG43 motif plays no role in DNase activity of Tde1 but is crucial for its protein stability. Tde1 with substitution of one of the two glycine residues to leucine retains stability of intact proteins but Tde1 is prone for truncations and degradation when both glycine residues are substituted to leucine. The instability is most evident when ectopically expressed in *E. coli* and when retaining DNase activity. Single glycine substitution (Tde1G39L and Tde1G43L variants) does not influence protein stability...
may suggest that the adjacent glycine residues (G35 or G47) are sufficient to compensate the loss of one glycine of G39xxxG43 motif for structural integrity in both variants. The importance of G39xxxG43 motif in Tde1 protein stability is consistent with the role of glycine zippers in structural impact 17.

With the knowledge of effector translocation mechanisms, the bacterial protein secretion apparatus also offers a strategy for use of effector translocation domain for delivering heterologous proteins to specific cells. T6SS is a promising vehicle for such purpose because effectors or secreted proteins appear to be delivered with their folded form, unlike those to be transported as unfolded forms in most of other specialized secretion systems 1. Engineering T6SS carriers such as VgrG spikes to carry exogenous effectors proteins into target cells are feasible but challenging 43-45. By using a truncated variant of PAAR, a recent study showed delivering exogenous T6SS effectors and Cre recombinase for genetic modification in the target cells 46. Our success in using N-Tde1 in the delivery of sfGFP proteins into target E. coli cells where they exert fluorescence also suggested potential applications of N-Tde1 for the delivery of proteins of interests such as genetic modifiers. This strategy provides advantages over transforming foreign DNA for expressing a protein of interest from creating undesired genome manipulation.

MATERIALS AND METHODS

Strains and growth conditions

The strains and plasmids used in this study are listed in the supplementary table (S1) and (S2). The E. coli strains used in this study are BW25113 and DH10B. All the A. tumefaciens strains were cultured on 523 medium 47 at 28 °C unless stated. The E. coli strains were cultured on Luria Bertani (LB) medium (10 g L−1 NaCl, 10 g L−1 tryptone, and 5 g L−1 yeast extract) at 37°C unless stated. Where appropriate, the media were supplemented with 100 µg mL−1 spectinomycin, gentamycin 25 µg mL−1 (for E. coli) and 50 µg mL−1 (for A. tumefaciens), 50 µg mL−1 ampicillin, 50 µg mL−1 kanamycin, 1 mM Isopropyl β- d-1-thiogalactopyranoside (IPTG).

Growth inhibition assay
For growth inhibition assay of IPTG-inducible expression of Tde1 and its variants, *E. coli* (DH10B) harbouring pTrc200 vector or the derivatives expressing Tde1 variants were grown overnight in LB medium supplemented with spectinomycin prior to 1:30 dilution in a fresh medium and incubated for 2 hrs at 37°C with 250 rpm. After 2 hrs, the cultures were normalized to OD$_{600}$ 0.1 in LB with 1 mM IPTG for continuous culture in the same growth condition. The growth of *E. coli* was monitored for OD$_{600}$ every one hr using ULTROSPEC® 10 cell density meter (Biochrom, UK). For growth inhibition assay of arabinose-inducible expression, *E. coli* BW25113 harbouring pJN105 vector or the derivatives expressing Tde1 variants were used. Overnight cultures of *E. coli* cells were adjusted to an OD$_{600}$ of 0.1 in 200 μL LB with 0.2% L-arabinose in a 96-well plate. The OD$_{600}$ values were measured by the Synergy H1 microplate reader (Agilent Technologies, USA) with agitation at 37 °C. The OD$_{600}$ values of final time points were used to calculate mean ± SD of three repeats. One way Analysis of Variance (ANOVA) was used for the analysis of statistical significance followed by the Tukey’s multiple comparison.

**In vivo plasmid DNA degradation assay.** The *in vivo* plasmid DNA degradation assay was performed as described $^{23}$ with minor modifications. Briefly, overnight cultures of *E. coli* BW25113 carrying pJN105 vector or the derivatives expressing Tde1 variants were adjusted to an OD$_{600}$ of 0.3 in 4 mL LB with 0.5% D-glucose or 0.2% L-arabinose. After induction for 3 hrs, bacterial cells normalized by OD$_{600}$ were collected for plasmid DNA extraction and western blot analysis. The plasmids were then extracted and applied to 0.6% agarose gel electrophoresis to detect the DNA degradation. The OD$_{600}$ values were measured by DEN-600 photometer (Biosan, Latvia) every hr.

**β-galactosidase and viability assays for ONPG update**

β-galactosidase assay was performed as described $^{48}$ with minor modifications. BW25113 wild type, BW25113ΔlacY(pYTA-lacZ), or BW25113ΔlacY harbouring pTrc200 vector or the derivatives expressing Tde1 variants were grown overnight and refreshed to a fresh medium as stated for growth inhibition assay. After subculture for 2 hrs, the cells were induced with 1 mM IPTG, and incubated for one more hr. Part of the culture was adjusted to OD$_{600}$ = 0.3 in Z-buffer and the Intracellular β-galactosidase
activity was measured by mixing 100 µl of 4 mg/ml ONPG with 900 µL of the cells and incubation at room temperature for 10 mins prior to measurement at OD_{420}. The remaining cells were normalized to OD_{600} 0.3 in 0.9 % sterile saline and after serial dilution, 10 µL was spotted on LB plate without antibiotics to recover the viable cells. Data of OD_{420} were used to calculate mean ± SD of three independent experiments. One way ANOVA was used for the analysis of statistical significance followed by the Tukey's multiple comparison.

**Co-immunoprecipitation**

The Co-immunoprecipitation (co-IP) was performed according to the manufacturer's recommendations of EZview red Anti-HA agarose (Sigma-E6779) with minor modifications. To identify Tap1 interacting domain of Tde1, the HA tagged Tde1 variants were expressed on pTrc200 plasmid. For co-IP in *A. tumefaciens*, C58 Δtde1 cells expressing the Tde1 variants grown in 523 medium overnight were resuspended in a 1:30 ratio to a fresh medium and incubated at 25°C for 3 hrs followed by 1 mM IPTG induction for additional 3 hrs. After 6 hrs post incubation, the cells were normalized to OD_{600} of 5 per mL in ice-cold PBS buffer (pH 7.4). After cell lysis by lysozyme treatment and sonication, the lysate was centrifuged and a 100 µL aliquot of the lysate was saved for the input fraction. The remaining 900 µL lysate was mixed with 25 µL of pre-equilibrated EZview red Anti-HA agarose and incubated at 4 °C for 1 hr. The beads were then washed 3 times with ice-cold PBS buffer and the proteins bound to the beads were eluted with 100 µL of 2X SDS sample loading buffer. Similarly, the aliquoted input fraction was mixed with equal volume of 2X SDS sample loading buffer for analysis by western blotting.

For Co-IP in *E. coli*, the HA tagged Tde1 variants were expressed in DH10B cells from pTrc200 plasmid and Tap1 was expressed from pRL662 plasmid. Overnight culture was resuspended to fresh medium with 1:30 ratio and incubated for two hrs, followed by induction with 1 mM IPTG and incubated at 37°C with 250 rpm for two hrs. The cells were lysed followed by co-IP as stated earlier.

**Secretion assay**
Type VI Secretion assay was performed in 523 as described previously. Briefly, A. *tumefaciens* strain was cultured overnight in 523 medium and normalized to OD$_{600}$ of 0.2 in a fresh medium. After 6 hrs of culture, the secreted proteins were collected by centrifuging at 10,000 g for 5 mins. The resulting pellet was adjusted to OD$_{600}$ of 10 as a cellular fraction. The culture supernatant was filtered with 0.22 μm Millipore filter membrane and resulting filtrate was subjected for TCA precipitation and referred as an extracellular fraction.

**Western blotting**

Western blot analyses were done as previously described. The following primary antibody titres used were: HA epitope (1:4,000), Tap1 (1:3,000), Strep (1:4,000), EF-Tu (1:6,000), and C-terminal Tde1 (1:4,000) $^{23,28}$, Hcp (1:2,500) $^{49}$.

**Interbacterial competition assays**

For interbacterial competition with *E. coli* (target), A. *tumefaciens* strain (attacker) was grown overnight at 28°C in 523 broth with appropriate antibiotics if needed. *E. coli* DH10B harbouring pRL662 plasmid was grown at 37°C in LB broth with gentamycin. After harvesting and washing the cells in 0.9% saline, the attacker to target cell density was adjusted to 30:1 (OD$_{600}$ = 3: 0.1) and the mix was spotted on Agrobacterium kill-triggering (AK) medium containing 1.5% (wt/vol) agar $^{24}$. Similar procedure is used when using A. *tumefaciens* strain 1D1609 as a target, which was grown at 28°C in 523 broth prior to competition. After incubation of the mixed strains for 16 hrs at 28°C, the surviving cells were selected on a gentamycin-containing LB agar square plate.

**Fluorescence microscopy**

For propidium iodide and Hoechst staining, *E. coli* cells (BW25113) harbouring Trc200 vector or derivatives expressing Tde1 variants were grown overnight and refreshed to a fresh medium as stated for growth inhibition assay. After subculture for 2 hrs, the cells were induced with 1 mM IPTG for one hr and OD$_{600}$ equivalent to 0.3 was collected in 1 ml PBS and stained with Hoechst 33342 (H3570) to a final concentration of 12.3 μg/mL and 1 μg/mL Propidium iodide (2208511) and incubated for 2 minutes in dark. Finally, 2 μL was spotted on 2.5% agarose pad.
For the translocation experiment, the sfGFP fused Tde1 variants were expressed in A. *tumefaciens* ∆tdei cells (attacker). *E. coli* (target) cells were labelled with mCherry (false colour blue) expressed from pBBRMCS2. *A. tumefaciens* attacker cells were cultured in 523 broth overnight, and *E. coli* target cells were separately cultured on LB broth. Overnight cultured attacker and target cells were mixed at 5:1 ratio (OD<sub>600</sub> = 1.0: 0.2), and 10 µL of the mix was cultured on LB-agar plate without IPTG. After 20 hrs of co-culture, the cells were washed with 100 µL PBS and 2 µL of suspension was spotted on the 2.5% agarose pad on a microscopic slide. The merged GFP and mCherry signals was based on a cyan fluorescence.

Fluorescence microscopy was performed using Axio Observer 7 (Zeiss, Germany) microscope equipped with an Axiochem 702 digital camera and a Plan-Apochromat 100x/1.4 Oil DIC H objective. Exposure times were adjusted to 20 ms for Phase, 20 ms for Hoechst, 150 ms for PI, 200 ms for GFP and 5000 ms for mCherry. Multiple images were taken from different fields and all the experiments were performed at least in triplicate and a representative image is shown. Images were analyzed by using ZEN 2.3 (blue edition) software.

### Domain prediction and analysis

Full-length Tde1 (1-278) was used as a query for conserved domain search on the conserved domains database (CDD) of the National Center for Biotechnology Information (NCBI). Prediction of transmembrane domain was done using the PRED-TMR2. The Tde1 homologues and tape measure proteins (TMPs) for the multiple sequence alignment were obtained by BLAST search of N-Tde1 (1-97) against the NCBI non redundant database (nr) with representative sequences selected for multiple sequence alignment. The domain architectures of the Ntox15 domain containing proteins were obtained using the full length Tde1 against Conserved Domain Architectural retrieval tool (CDART) of NCBI. The information of gene clusters encoding Tde1 homologues and TMPs including upstream and downstream three genes was retrieved from their respective genomes. Full-length Tde1 (1-278) was used as a query for structural prediction on a Phyre2. Three-dimensional structure modelling was done.
using Phyre2 in intensive modelling mode. Crystal structure served as best template for
the N-terminus and percentage of confidence for three-dimensional structure modelling
are indicated in the legends of corresponding figures. The structural graphics were
generated by using ChimeraX 1.1.

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Microbial Biology, Academia Sinica. The authors also thank Ying Wang for illustration of
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interpretation, or the decision to submit the work for publication.

AUTHOR CONTRIBUTION

Conceptualization: JA, MY, EML; Investigation: JA, MY, LKS, YWC; Project Funding
acquisition and administration: EML; Supervision: EML; Writing of original draft: JA,
EML; Writing – methodology, review & editing: JA, MY, LKS, YWC, EML.

FIGURE LEGENDS

Figure 1. Schematic domain organization, sequence alignment, growth inhibition
assay. (A) Schematic domain organization of Tde1 protein and its variants. The N-
terminal repeated glycine zipper motifs (12-51) overlapping a predicted transmembrane domain (22-42) and Ntox15 DNase domain (99-247) are indicated. Tde1 and its variants with truncation or amino acid substitutions were illustrated. (B) Growth inhibition assay of E. coli DH10B cells harbouring pTrc200 vector or each of its derivatives expressing Tde1 variants with IPTG induction. (C) Growth inhibition assay of E. coli DH10B cells co-expressing the Tde1 variants expressed from pTrc200 plasmid and Tdi1 immunity gene expressed from pRL662 plasmid. Growth curve was determined at OD600. Graphs of panels B and C show mean ± SD of three independent experiments. The significant differences were shown by the different letters (p value <0.01). (D) Multiple sequence alignment of N-Tde1 homologues were presented with highly conserved amino acid residues highlighted in yellow. The bacterial species, strain name, and locus number of Tde1 orthologs (Agrobacterium/Rhizobium) or tape measure proteins (Paraburkholderia/Burkholderia) are indicated on the left and right of aligned sequences. Two conserved glycine residues (G39, G43) subjected for mutagenesis were indicated by the arrows above the sequences.

Figure 2. Membrane permeabilization assays. (A) Growth inhibition assay of E. coli DH10B cells harbouring pTrc200 vector or each of its derivatives expressing Tde1 variants with IPTG inducible expression. BW25113 WT or BW25113 (∆lacY) cells harbouring pTrc200 vector or each of its derivatives expressing Tde1 variants were carried out for (B) β-galactosidase activity assay to determine ONPG uptake, (C) propidium iodide permeability with cells treated with Propidium iodide and Hoechst for detection by fluorescence microscope, and (D) viability assay for E. coli cells derived from the ONPG uptake assay. (E) Bacteriostatic activity assay. E. coli DH10B cells harbouring pTrc200 vector or each of its derivatives expressing Tde1 variants were cultured with or without IPTG induction for 1 hr. The IPTG-induced cells were further centrifuged and resuspended in the fresh medium with or without IPTG. Cell density was measured again before continuous growth for additional 1 hr. Graphs of panels A, B, and E show mean ± SD of three independent experiments. The significant differences were shown by the
different letters (p value <0.01). Panels C and D are representative results of three independent experiments.

Figure 3. The N-terminus of Tde1 is sufficient for interaction with Tap1. (A) Co-immunoprecipitation (Co-IP) in A. tumefaciens. A. tumefaciens C58 Δtde1 harbouring pTrc200 vector or its derivatives expressing HA-tagged Tde1 variants. Anti-HA resin was used to co-IP the Tde1 variants and Tap1. (B) Co-IP in E. coli DH10B. DH10B cells co-expressing HA-tagged Tde1 variants from pTrc200 and Tap1 from pRL662 were collected for co-IP. Anti-HA resin was used to co-IP the Tde1 variants and Tap1. Representative western blot results of three independent experiments were shown with antibody against HA for Tde1 variants and Tap1 respectively. (C). Secretion assay for HA-tagged Tde1 variants. Western blot for the cellular and extracellular fractions of A. tumefaciens C58 Δtde1 and Δtde1ΔtssK expressing the HA-tagged Tde1 variants. Hcp secretion was detected as a positive control for active T6SS secretion. Representative western blot results of three independent experiments were shown with antibody against HA, Hcp, or EF-Tu serving as a loading and non-secreted protein control. Protein markers are indicated in kDa.

Figure 4. The roles of N-Tde1 and G^{39}xxxG^{43} glycine zipper motif for secretion and translocation. (A) Secretion assay for Tde1 variants fused with sfGFP. Western blot for the cellular and extracellular fractions of A. tumefaciens C58 Δtde1 and Δtde1ΔtssK expressing the Tde1 variants fused with sfGFP were detected by anti-GFP antibody. Representative western blot results of three independent experiments were shown with antibody against GFP, Hcp, or EF-Tu serving as a loading and non-secreted protein control. Hcp secretion served as a positive control for active T6SS secretion. Protein markers are indicated in kDa. (B) Fluorescence microscopy for Tde1 translocation. A. tumefaciens C58 Δtde1 expressing Tde1 variants fused with sfGFP (in green) and E. coli DH10B carrying mCherry (false coloured in blue) were co-cultured for 20 hrs. A cyan fluorescence with merged blue and green signals represented the translocation of Tde1 variants from A. tumefaciens to E. coli.
Figure 5. G^{39}xxxG^{43} glycine zipper motif of Tde1 is required for DNase-mediated killing of target cells during interbacterial competition. (A) Inter-species Interbacterial competition. A. tumefaciens C58 Δtdei and ΔtdeiΔtssK expressing the Tde1 variants were co-incubated with E. coli (DH10B) cells on AK medium followed by selecting target cell survival with serial dilution on a gentamycin containing LB agar at 37°C. (B) Intra-species Interbacterial competition between A. tumefaciens C58 (Δtdei) and ΔtdeiΔtssK expressing the Tde1 variants and A. tumefaciens 1D1609 target cell. The competition was done as in (B) except 1D1609 was recovered at 28°C. (C) Secretion assay for Tde1 and its variants co-expressed with its immunity protein Tdi1. The toxin immunity pairs Tde1-Tdi1, Tde1^{GLGL}-Tdi1, and Tde1(M)-Tdi1 were expressed in A. tumefaciens C58 Δtdei and ΔtdeiΔtssK. (D) In vivo plasmid DNA degradation assay. E. coli BW25113 carrying pJN105 empty vector or the derivatives expressing different variants of Tde1 were supplemented with 0.5% glucose (“-”) or 0.2% L-arabinose (“+”) for 3 hrs to either repress or induce Tde1 production. The plasmids were then extracted to observe the DNA degradation and bottom panel showed western blots of specific Tde1 protein bands indicated by arrows. (E) Growth inhibition assay of Tde1 and its variants. E. coli BW25113 cells were induced by adding 0.2% L-arabinose for Tde1 production. The OD_{600} values were measured every 15 minutes. The OD_{600} values of the 4 h post L-arabinose induction were analysed for statistical analysis. Graphs show mean ± SD of three biological repeats. The significant differences were shown by the different letters (p value <0.01). All data were shown with representative results of three independent experiments. Western blots were detected with specific antibody against Tde1, Hcp, or EF-Tu serving as a loading and non-secreted protein control. Protein markers are indicated in kDa.

Figure 6. Proposed model of the loading, firing, and translocation of Tde1. The Tde1 translocation is proposed through six steps. Step 1: Tde1 forms a complex with Tdi1 and Tap1 in the attacker cell. Step 2: Tap1-Tde1-Tdi1 complex binds to the VgrG and the Hcp-VgrG-PAAR puncturing device carrying Tde1 effector complex is loaded onto
membrane-associated baseplate. Step 3: Hcp tube and TssB/C sheath polymerize on the Tde1-loaded VgrG/baseplate while Tdi1 and Tap1 fall off with unknown mechanisms before or upon firing. Step 4: TssBC sheath contracts and ejects Tde1 into the target cell periplasm or cytoplasm. Step 5: The glycine zipper(s) on the N-terminus of Tde1 permeabilize the target cell membrane. Step 6: Intact or truncated Tde1 proteins attack DNA for degradation in the target cell.

**Figure S1. Domain architecture and genetic organizations of Tde homologues.** (A) Domain organization of the Ntox15-containing proteins. Top 10 classes of the Ntox15-containing proteins are shown with the identifiable domains (not to scale). The number of proteins in each class were indicated on the left based on the information on June 29, 2022. The *A. tumefaciens* Tde1 belonged to the first class where the N-terminal region lacks an identifiable domain. (B) Genetic organizations of genes encoding Tde1 orthologues and Tape Measure Proteins (TMPs) with sequence similarity to N-terminus of Tde1. The proteins encoded from the upstream and downstream of *tde1* and *tmp* genes are shown with their identified domain organizations.

**Figure S2. Growth inhibition assays in *A. tumefaciens* and *E. coli*.** Growth curve (A) and western blot analyses (B) of *A. tumefaciens* C58 Δtde1 carrying pTrc200 or its derivatives expressing HA-tagged Tde1 variants. The growth curve was detected every 2 hrs in 523 medium supplemented with 1 mM IPTG. Graphs show mean ± SD of three independent experiments. The significant differences were shown by the different letters (p value <0.01). The proteins collected at end point (6 hr) were analysed for western blotting with antibody against HA. Representative results of three independent experiments were shown. Protein markers are indicated in kDa. (C) The growth curve analysis of *E. coli* cells used for *in vivo* plasmid DNA degradation assay. The turbidity of *E. coli* BW25113 expressing Tde1 and its variants carried out for the *in vivo* plasmid DNA degradation assay were measured. The *E. coli* cells were supplemented with 0.5% glucose (glu) or 0.2% L-arabinose (ara) for the repression or induction of Tde1 and its
variants. The OD$_{600}$ values were measured by DEN-600 photometer (Biosan, Latvia) every hr.

**Figure S3.** Fluorescence microscopy for negative controls of translocation assay. *A. tumefaciens* C58 $\Delta$tde1$\Delta$tssK expressing N-Tde1-sfGFP or Tde1(M)-sfGFP (in green) and *E. coli* DH10B carrying mCherry (false coloured in blue) were co-cultured for 20 hrs. No cyan fluorescence with merged blue and green signals could be detected when attacker cells are T6SS-inactive, which served as negative controls for the translocation assay.

**Figure S4.** Structural prediction of the Tde1 N-terminus with similarity to pyocin S5 and colicins. (A) Predicted results of N-terminal Tde1 (1-97) as a query. (B) N-terminal Tde1 with structural similarity to pore-forming domain of the pyocin S5, colicines and other membrane perturbing proteins based on Phyre2 prediction. (C) Cartoon model of the Tde1 (residue 10 – 62) by using on the basis of the crystal structure of Pyocin S5 (PDB 6THK) with 77.3% of identity. Glycine residues of Tde1 were indicated. (D) Superimposition of N-Tde1 and pore-forming domain of pyocin S5. Tde1 N-terminus is in red and partially pore-forming domain of pyocin S5 is in teal, G$^{39}$ and G$^{43}$ in the putative glycine zipper motif are highlighted in green. All data were analyzed by Phyre2 server.

**REFERENCES**


Glycine zipper mediated effector entry


### Table S1. List of Strains and plasmids

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<td>pJN105_Tde1&lt;sub&gt;GLGL&lt;/sub&gt;</td>
<td>Gm&lt;sup&gt;R&lt;/sup&gt;, pJN105 expressing C-terminal 6xHis tagged Tde1 with the substitutions of G39L and G43L</td>
<td>This study</td>
<td></td>
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<tr>
<td>pJN105_Tde1(M)</td>
<td>Gm&lt;sup&gt;R&lt;/sup&gt;, pJN105 expressing C-terminal 6xHis tagged Tde1 with the substitutions of H190A and D193A</td>
<td>This study</td>
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## Table S2. List of primers used to make constructs

<table>
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<tr>
<th>Plasmid/Construct made with the primers</th>
<th>Primer names and Sequence (restriction site is indicated underline)</th>
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<td>pTrc200_sfGFPP</td>
<td>Ncol_sfGFP_F: TGGACCCATGGGACCCGTAAAGGCAGAGCTTGTTCA</td>
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<td>HindIII_sfGFP_R: TCGACAAGCTTTTTGTACAGTTCCATCCATACG</td>
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<td>Ncol_sfGFP_F: TGGACCACATGGACCGTAAAGGCAGAGCTTGTTCA</td>
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<td>HindIII_sfGFP_R: TCGACAAGCTTTTTGTACAGTTCCATCCATACG</td>
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<td>pET-R1-Sacl: GGAAGAGCTCCTTTCCGGCGTGTAG</td>
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A. Schematic domain organization of Tde1 protein and its variants. The N-terminal repeated glycine zipper motifs (12-51) overlapping a predicted transmembrane domain (22-42) and Ntox15 DNase domain (99-247) are indicated. Tde1 and its variants with truncation or amino acid substitutions were illustrated. (B) Growth inhibition assay of E. coli DH10B cells harbouring pTrc200 vector or each of its derivatives expressing Tde1 variants with IPTG induction. (C) Growth inhibition assay of E. coli DH10B cells co-expressing the Tde1 variants expressed from pTrc200 plasmid and Tdi1 immunity gene expressed from pRL662 plasmid. Growth curve was determined at OD600. Graphs of panels B and C show mean ± SD of three independent experiments. The significant differences were shown by the different letters (p value <0.01). (D) Multiple sequence alignment of N-Tde1 homologues were presented with highly conserved amino acid residues highlighted in yellow. The bacterial species, strain name, and locus number of Tde1 orthologs (Agrobacterium/Rhizobium) or tape measure proteins (Paraburkholderia/Burkholderia) are indicated on the left and right of aligned sequences. Two conserved glycine residues (G39, G43) subjected for mutagenesis were indicated by the arrows above the sequences.

Figure 1. Schematic domain organization, sequence alignment, growth inhibition assay. (A) Schematic domain organization of Tde1 protein and its variants. The N-terminal repeated glycine zipper motifs (12-51) overlapping a predicted transmembrane domain (22-42) and Ntox15 DNase domain (99-247) are indicated. Tde1 and its variants with truncation or amino acid substitutions were illustrated. (B) Growth inhibition assay of E. coli DH10B cells harbouring pTrc200 vector or each of its derivatives expressing Tde1 variants with IPTG induction. (C) Growth inhibition assay of E. coli DH10B cells co-expressing the Tde1 variants expressed from pTrc200 plasmid and Tdi1 immunity gene expressed from pRL662 plasmid. Growth curve was determined at OD600. Graphs of panels B and C show mean ± SD of three independent experiments. The significant differences were shown by the different letters (p value <0.01). (D) Multiple sequence alignment of N-Tde1 homologues were presented with highly conserved amino acid residues highlighted in yellow. The bacterial species, strain name, and locus number of Tde1 orthologs (Agrobacterium/Rhizobium) or tape measure proteins (Paraburkholderia/Burkholderia) are indicated on the left and right of aligned sequences. Two conserved glycine residues (G39, G43) subjected for mutagenesis were indicated by the arrows above the sequences.
Figure 2. Membrane permeabilization assays. (A) Growth inhibition assay of *E. coli* DH10B cells harbouring pTrc200 vector or each of its derivatives expressing Tde1 variants with IPTG inducible expression. BW25113 WT or BW25113 (∆lacY) cells harbouring pTrc200 vector or each of its derivatives expressing Tde1 variants were carried out for (B) β-galactosidase activity assay to determine ONPG uptake, (C) propidium iodide permeability with cells treated with Propidium iodide and Hoechst for detection by fluorescence microscope, and (D) viability assay for *E. coli* cells derived from the ONPG uptake assay. (E) Bacteriostatic activity assay. *E. coli* DH10B cells harbouring pTrc200 vector or each of its derivatives expressing Tde1 variants were cultured with or without IPTG induction for 1 hr. The IPTG-induced cells were further centrifuged and resuspended in the fresh medium with or without IPTG. Cell density was measured again before continuous growth for additional 1 hr. Graphs of panels A, B, and E show mean ± SD of three independent experiments. The significant differences were shown by the different letters (p value <0.01). Panels C and D are representative results of three independent experiments.
Figure 3. The N-terminus of Tde1 is sufficient for interaction with Tap1. (A) Co-immunoprecipitation (Co-IP) in A. tumefaciens. A. tumefaciens C58 Δtde1 harbouring pTrc200 vector or its derivatives expressing HA-tagged Tde1 variants. Anti-HA resin was used to co-IP the Tde1 variants and Tap1. (B) Co-IP in E. coli DH10B. DH10B cells co-expressing HA-tagged Tde1 variants from pTrc200 and Tap1 from pRL662 were collected for co-IP. Anti-HA resin was used to co-IP the Tde1 variants and Tap1. Representative western blot results of three independent experiments were shown with antibody against HA for Tde1 variants and Tap1 respectively. (C). Secretion assay for HA-tagged Tde1 variants. Western blot for the cellular and extracellular fractions of A. tumefaciens C58 ∆tde1 and ∆tde1 ∆tssK expressing the HA-tagged Tde1 variants. Hcp secretion was detected as a positive control for active T6SS secretion. Representative western blot results of three independent experiments were shown with antibody against HA, Hcp, or EF-Tu serving as a loading and non-secreted protein control. Protein markers are indicated in kDa.
Figure 4. The roles of N-Tde1 and G<sup>39</sup>G<sup>43</sup> glycine zipper motif for secretion and translocation. (A) Secretion assay for Tde1 variants fused with sfGFP. Western blot for the cellular and extracellular fractions of A. tumefaciens C58 Δtdei and Δtdei1ΔtssK expressing the Tde1 variants fused with sfGFP were detected by anti-GFP antibody. Representative western blot results of three independent experiments were shown with antibody against GFP, Hcp, or EF-Tu serving as a loading and non-secreted protein control. Hcp secretion served as a positive control for active T6SS secretion. Protein markers are indicated in kDa. (B) Fluorescence microscopy for Tde1 translocation. A. tumefaciens C58 Δtdei expressing Tde1 variants fused with sfGFP (in green) and E. coli DH10B carrying mCherry (false coloured in blue) were co-cultured for 20 hrs. A cyan fluorescence with merged blue and green signals represented the translocation of Tde1 variants from A. tumefaciens to E. coli.
A glycine zipper motif of Tde1 is required for DNase-mediated killing of target cells during interbacterial competition. (A) Interspecies interbacterial competition. *A. tumefaciens* C58 Δtdei and Δtdei1ΔtssK expressing the Tde1 variants were co-incubated with *E. coli* (DH10B) cells on AK medium followed by selecting target cell survival with serial dilution on a gentamycin containing LB agar at 37°C. (B) Intra-species interbacterial competition between *A. tumefaciens* C58 (Δtdei) and Δtdei1ΔtssK expressing the Tde1 variants and *A. tumefaciens* 1D1609 target cell. The competition was done as in (B) except 1D1609 was recovered at 28°C. (C) Secretion assay for Tde1 and its variants co-expressed with its immunity protein Tdi1. The toxin immunity pairs Tde1-Tdi1, Tde1GLGL-Tdi1, and Tde1(M)-Tdi1 were expressed in *A. tumefaciens* C58 Δtdei and Δtdei1ΔtssK. (D) In vivo plasmid DNA degradation assay. *E. coli* BW25113 carrying pJN105 empty vector or the derivatives expressing different variants of Tde1 were supplemented with 0.5% glucose (“-”) or 0.2% L-arabinose (“+”) for 3 hrs to either repress or induce Tde1 production. The plasmids were then extracted to observe the DNA degradation and bottom panel showed western blots of specific Tde1 protein bands indicated by arrows. (E) Growth inhibition assay of Tde1 and its variants. *E. coli* BW25113 cells were induced by adding 0.2% L-arabinose for Tde1 production. The OD600 values were measured every 15 minutes. The OD600 values of the 4 h post L-arabinose induction were analysed for statistical analysis. Graphs show mean ± SD of three biological repeats. The significant differences were shown by the different letters (p value <0.01). All data were shown with representative results of three independent experiments. Western blots were detected with specific antibody against Tde1, Hcp, or EF-Tu serving as a loading and non-secreted protein control. Protein markers are indicated in kDa.
Figure 6. Proposed model of the loading, firing, and translocation of Tde1. The Tde1 translocation is proposed through six steps. Step 1: Tde1 forms a complex with Tdi1 and Tap1 in the attacker cell. Step 2: Tap1-Tde1-Tdi1 complex binds to the VgrG and the Hcp-VgrG-PAAR puncturing device carrying Tde1 effector complex is loaded onto membrane-associated baseplate. Step 3: Hcp tube and TssB/C sheath polymerize on the Tde1-loaded VgrG/baseplate while Tdi1 and Tap1 fall off with unknown mechanisms before or upon firing. Step 4: TssBC sheath contracts and ejects Tde1 into the target cell periplasm or cytoplasm. Step 5: The glycine zipper(s) on the N-terminus of Tde1 permeabilize the target cell membrane. Step 6: Intact or truncated Tde1 proteins attack DNA for degradation in the target cell.
Figure S1. Domain architecture and genetic organizations of Tde homologues. (A) Domain organization of the Ntox15-containing proteins. Top 10 classes of the Ntox15-containing proteins are shown with the identifiable domains (not to scale). The number of proteins in each class were indicated on the left based on the information on June 29, 2022. The A. tumefaciens Tde1 belonged to the first class where the N-terminal region lacks an identifiable domain. (B) Genetic organizations of genes encoding Tde1 orthologues and Tape Measure Proteins (TMPs) with sequence similarity to N-terminus of Tde1. The proteins encoded from the upstream and downstream of tde1 and tmp genes are shown with their identified domain organizations.
Figure S2. Growth inhibition assays in *A. tumefaciens* and *E. coli*. Growth curve (A) and western blot analyses (B) of *A. tumefaciens* C58 Δtde1 carrying pTrc200 or its derivatives expressing HA-tagged Tde1 variants. The growth curve was detected every 2 hrs in 523 medium supplemented with 1 mM IPTG. Graphs show mean ± SD of three independent experiments. The significant differences were shown by the different letters (p value <0.01). The proteins collected at end point (6 hr) were analysed for western blotting with antibody against HA. Representative results of three independent experiments were shown. Protein markers are indicated in kDa. (C) The growth curve analysis of *E. coli* cells used for *in vivo* plasmid DNA degradation assay. The turbidity of *E. coli* BW25113 expressing Tde1 and its variants carried out for the *in vivo* plasmid DNA degradation assay were measured. The *E. coli* cells were supplemented with 0.5% glucose (glu) or 0.2% L-arabinose (ara) for the repression or induction of Tde1 and its variants. The OD600 values were measured by DEN-600 photometer (Biosan, Latvia) every hr.
Figure S3. Fluorescence microscopy for negative controls of translocation assay. A. tumefaciens C58 Δtde1ΔtssK expressing N-Tde1-sfGFP or Tde1(M)-sfGFP (in green) and E. coli DH10B carrying mCherry (false coloured in blue) were co-cultured for 20 hrs. No cyan fluorescence with merged blue and green signals could be detected when attacker cells are T6SS-inactive, which served as negative controls for the translocation assay.
Figure S4. Structural prediction of the Tde1 N-terminus with similarity to pyocin S5 and colicins. (A) Predicted results of N-terminal Tde1 (1-97) as a query. (B) N-terminal Tde1 with structural similarity to pore-forming domain of the pyocin S5, colicines and other membrane perturbing proteins based on Phyre2 prediction. (C) Cartoon model of the Tde1 (residue 10 – 62) by using on the basis of the crystal structure of Pyocin S5 (PDB 6THK) with 77.3% of identity. Glycine residues of Tde1 were indicated. (D) Superimposition of N-Tde1 and pore-forming domain of pyocin S5. Tde1 N-terminus is in red and partially pore-forming domain of pyocin S5 is in teal, G^{19} and G^{43} in the putative glycine zipper motif are highlighted in green. All data were analyzed by Phyre2 server.