



Redundancy and Specificity of Type VI Secretion *vgrG* Loci in Antibacterial Activity of *Agrobacterium tumefaciens* 1D1609 Strain

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Type VI secretion system (T6SS) is a contractile nanoweapon employed by many Proteobacteria to deliver effectors to kill or inhibit their competitors. One T6SS gene, *vgrG*, encodes a spike protein for effector translocation and is often present as multiple copies in bacterial genomes. Our phylogenomic analyses sampled 48 genomes across diverse Proteobacteria lineages and found ~70% of them encode multiple *VgrGs*, yet only four genomes have nearly identical paralogs. Among these four, *Agrobacterium tumefaciens* 1D1609 has the highest *vgrG* redundancy. Compared to *A. tumefaciens* model strain C58 which harbors two *vgrG* genes, 1D1609 encodes four *vgrG* genes (i.e., *vgrGa-d*) with each adjacent to different putative effector genes. Thus, 1D1609 was selected to investigate the functional redundancy and specificity of multiple *vgrG* genes and their associated effectors. Secretion assay of single and multiple *vgrG* deletion mutants demonstrated that these four *vgrGs* are functionally redundant in mediating T6SS secretion. By analyzing various *vgrG* mutants, we found that all except for the divergent *vgrGb* could contribute to 1D1609's antibacterial activity. Further characterizations of putative effector-immunity gene pairs revealed that *vgrGa*-associated gene 2 (*v2a*) encodes an AHH family nuclease and serves as the major antibacterial toxin. Interestingly, C58's *VgrG2* shares 99% amino acid sequence identity with 1D1609's *VgrGa*, *VgrGc* and *VgrGd*. This high sequence similarity allows 1D1609 to use an exogenous *VgrG* delivered from C58 to kill another competing bacterium. Taken together, *Agrobacterium* can use highly similar *VgrGs*, either produced endogenously or injected from its close relatives, for T6SS-mediated interbacterial competition.

Keywords: type VI secretion system, *VgrG*, effector, antibacterial, functional redundancy, *Agrobacterium tumefaciens*

INTRODUCTION

Bacteria have evolved many survival strategies, including pathogenesis, competition, and cooperation, to thrive in diverse and changing environments. One of the weapons that they use is type VI secretion system (T6SS), a machinery that delivers effectors to both eukaryotic and prokaryotic target cells. Its coding genes are present in approximately 25% of the Gram-negative bacterial genomes sequenced (Bingle et al., 2008).

T6SS has an important role in interbacterial competition and provides T6SS-possessing bacteria with competitive advantage in microbial community. It can deliver a variety of effectors such as nuclease, amidase, phospholipase, peptidoglycan hydrolase, muramidase, glycosidase, NADase, and ADP-ribosyltransferase into the target bacterial cell by a contractile machinery (Russell et al., 2014; Tang et al., 2018; Ting et al., 2018). Contraction of the sheath-like structure drives the inner tube composed of Hcp tipped by VgrG-PAAR and the associated effectors across bacterial membranes to extracellular milieu or into the target cell. After firing, the structure is immediately disassembled into its individual components, which can be recycled to assemble new machinery for continuous firing (Zoued et al., 2014).

The spike component VgrG is homologous to the gp27/gp5 complex or the tail spike of bacteriophage T4 and assembles into a trimeric complex (Leiman et al., 2009). Current knowledge based on studies from several bacterial systems suggests that VgrG is specifically required for delivery of cognate effector(s) encoded in the same *vgrG* genetic module (Hachani et al., 2014; Whitney et al., 2014). Furthermore, the C-terminal variable region of VgrG is the molecular determinant conferring specificity of effector delivery by binding to its cognate effector directly or via adaptor/chaperone that interacts with a specific effector (Bondage et al., 2016; Flaugnatti et al., 2016). T6SS adaptors/chaperones including DUF4123-, DUF1795-, and DUF2169-containing proteins are required for loading a specific effector onto the cognate VgrG for delivery (Lien and Lai, 2017).

Agrobacterium tumefaciens is a soil Alphaproteobacterium that infects a broad range of dicotyledonous plants and transfers T-DNA, an oncogenic DNA fragment, to plant's nuclear chromosomes (Gelvin, 2000; Hwang et al., 2017). *A. tumefaciens* strain C58 encodes a single T6SS gene cluster and is equipped with three toxins namely type VI amidase effector (Tae), type VI DNase effector 1 and 2 (Tde1 and Tde2), in which its toxin activity can be neutralized by its cognate immunity. Tde is a major antibacterial weapon during *in planta* interbacterial competition and its associated VgrG is specifically required for Tde1/2-dependent bacterial killing (Ma et al., 2014; Bondage et al., 2016). A gene which encodes DUF2169 is always found between *vgrG2* and *tde2* orthologs across many Proteobacterial classes (Bondage et al., 2016). In *A. tumefaciens* strain C58, a DUF2169-containing protein encoded upstream of *tde2* is required to stabilize Tde2 and for Tde2-mediated antibacterial activity. For *vgrG1*-associated Tde1, Tap1 (encoded upstream of *tde1*) is the specific chaperone/adaptor interacting with Tde1 prior to loading to VgrG1 for formation of Tde1-Tap1-VgrG-PAAR secretion complex (Bondage et al., 2016).

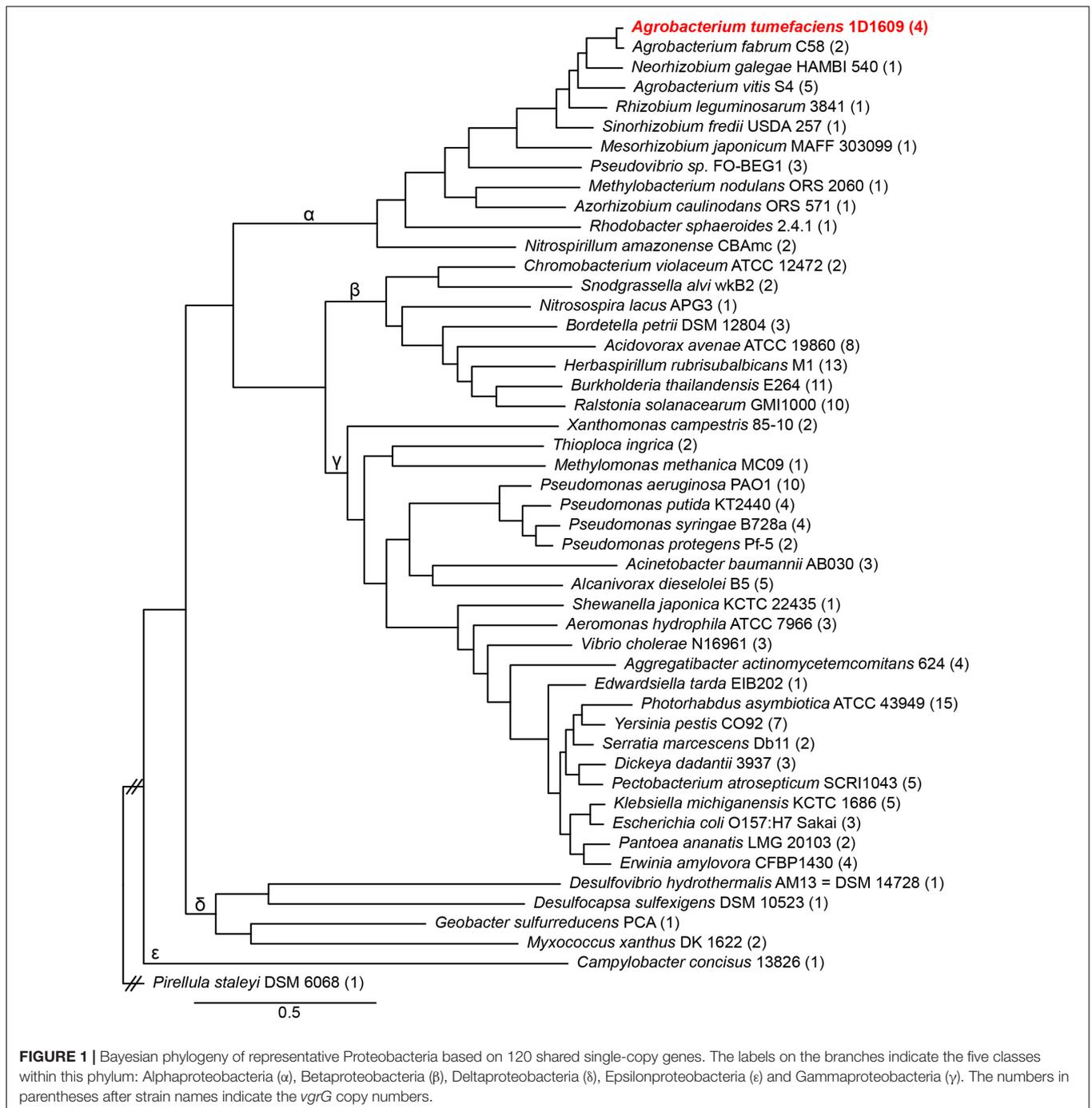
Aside from the representative *A. tumefaciens* model strain C58, there is little knowledge of T6SS available for other *A. tumefaciens* strains. Our recent comparative analysis of T6SS gene clusters from 11 *A. tumefaciens* strains with complete genome sequences revealed that T6SS is present in all sequenced strains belonging to different genomospecies (Wu et al., 2019b). The *imp* operon (*impA-N*) encoding core structural or regulatory components and the first five genes (*clpV*, *tai*, *tae*, *hcp*, *vgrG*) encoded in the *hcp* operon are highly conserved but the *vgrG*-associated downstream genes are variable. While all strains only harbor one *vgrG* gene encoded in the main T6SS gene cluster, additional orphan *vgrG* genes not genetically linked to the main T6SS gene cluster were often identified in some strains.

A T6SS-harboring bacterium can encode one to multiple VgrG proteins, in which several of them were demonstrated to be specifically required for delivery of cognate effector(s) encoded in the same *vgrG* genetic module (Hachani et al., 2014; Whitney et al., 2014). However, the prevalence and biological significance of *vgrG* redundancy has not been tackled. In this study, *A. tumefaciens* 1D1609 which has the highest number of nearly identical *vgrG* genes among all sampled Proteobacteria lineages was chosen to address this question. We investigated the functional redundancy and specificity of multiple VgrG in the effector delivery strategies of T6SS in the context of interbacterial competition. We generated single to multiple in-frame deletion of *vgrG* mutants to characterize the role of paralogous VgrG proteins and its associated effectors in 1D1609. We found that the four *vgrGs* are functionally redundant in mediating Hcp secretion but also exert both redundancy and specificity in mediating effector delivery for interbacterial competition. We also demonstrated that 1D1609 employs a nuclease effector as the major antibacterial toxin. Importantly, we provided experimental evidence that *A. tumefaciens* can use T6SS in the context of interbacterial competition to exchange VgrG as an effector carrier between its close siblings.

RESULTS

Majority of T6SS-Possessing Proteobacterial Genomes Harbor Multiple *vgrG* Genes

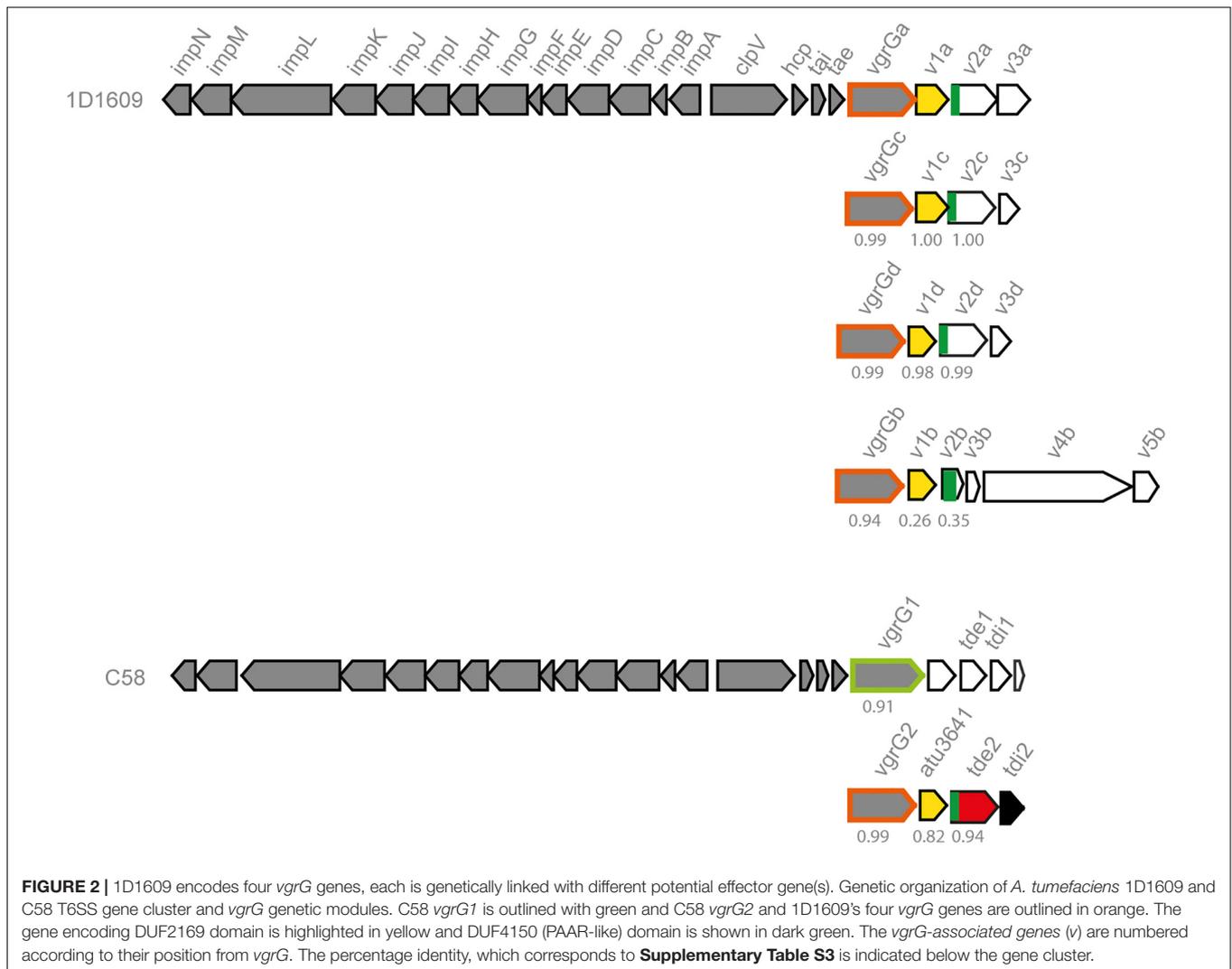
To survey the number of *vgrG* genes in T6SS-encoding bacterial genomes, 48 representative Proteobacteria harboring T6SS gene cluster(s) were selected for phylogenetic analysis. The result revealed that 33 of these genomes (~70%) encode multiple *vgrG* genes. The *vgrG* copy numbers have no strong tie to the phylogenetic placement of individual genomes (Figure 1), suggesting that the copy number evolution is highly dynamic, even at the intra-genus level (e.g., *Agrobacterium* in Alphaproteobacteria or *Pseudomonas* in Gammaproteobacteria). The *vgrG* gene tree (Supplementary Figure S1) is not congruent with the species tree (Figure 1) and the patterns suggest that horizontal gene transfers across different classes are not rare events. Nonetheless, the species with multiple VgrG homologs mostly (~64%) form a monophyletic clade at family levels.



The genome of all the sampled Rhizobiaceae lineages harbors only one main T6SS gene cluster and forms a monophyletic clade except for *Sinorhizobium fredii* (**Supplementary Figure S1**) (Bladergroen et al., 2003; Wu et al., 2019b). This pattern suggests that gene duplication could be the major driving force for copy number increase within families.

We further asked, when there are multiple *vgrG* homologs in a genome, how often can we find highly similar genes that are likely to be functionally redundant. Using protein sequence

identity $\geq 95\%$ as the cut-off, only four genomes met the criteria (**Supplementary Table S1**). These include Alphaproteobacterium *A. tumefaciens* strain 1D1609 (three out of four with 99% identity, the remaining one is 93–94% identical), Betaproteobacterium *Acidovorax avenae subsp. avenae* ATCC 19860 (two out of eight with 99% identity), Gammaproteobacterium *Aggregatibacter actinomycetemcomitans* strain 624 (four copies belonging to two types: within-type = 99–100% identity, between-type = 90% identity), and Gammaproteobacterium *Dickeya dadantii* strain



3937 (two out of three with 97% identity, the remaining one is 78% identical). Among them 1D1609 has the highest redundancy.

1D1609 Has Four *vgrG* Genes With Each Genetically Linked to Specific Putative Effector Gene(s)

1D1609 genome encodes one main T6SS gene cluster including *vgrGa* (At1D1609_RS23245) that is encoded in *hcp* operon within the T6SS main gene cluster. The other three *vgrGs* – *vgrGb* (At1D1609_RS26290), *vgrGc* (At1D1609_RS22460) and *vgrGd* (At1D1609_RS18895) are encoded in different loci elsewhere (**Figure 2**). All four *vgrG* genes are genetically linked with distinct potential effector gene(s) and associated genes predicted to function as adaptor/chaperone or immunity (Wu et al., 2019b). The four *vgrG* genetic modules consist of the first three genes with conserved domain and same gene order encoding VgrG-DUF2169-DUF4150. The *vgrGa*-, *vgrGc*-, and *vgrGd*-associated genes (*v2a*, *v2c*, *v2d*) encode N-terminal PAAR-like DUF4150 domain followed by C-terminal putative effector

domain. The *vgrGb*-linked *v2b* does not encode obvious effector domain and instead followed by downstream genes encoding putative effector domain, as predicted by Phyre or NCBI CDD search and BLASTP (Boratyn et al., 2013; Kelley et al., 2015; Marchler-Bauer et al., 2017) (**Supplementary Table S2**). The *vgrGc*-associated putative effector gene *v2c* does not encode any conserved domain, while the one (*v2d*) associated with *vgrGd* encodes a putative GH25 muramidase domain. Genes encoding DUF2169 domain are commonly found downstream of *vgrG* and upstream of DUF4150-containing effector genes. This gene order in *vgrG* genetic module is highly conserved in the *vgrG2* locus of C58, in which DUF2169-containing protein (Atu3641) may function as adaptor/chaperone of Tde2 due to its requirement for Tde2 stability and Tde2-dependent bacterial killing (Bondage et al., 2016). Thus, DUF2169-containing V1 protein is likely to function as a chaperone/adaptor required for delivery of cognate effectors in conjunction with the associated VgrG.

Unlike VgrG1 and VgrG2 which share 92% identity and are highly divergent at their C-terminus (Bondage et al., 2016),

we found that the three VgrG proteins – VgrGa, VgrGc and VgrGd in 1D1609 share 99% overall amino acid sequence identity (and 100% identity in the C-terminal region). These are also highly similar with VgrG2 of C58 (**Figure 2** and **Supplementary Table S3**). VgrGb shares 93–94% overall identity to the other three VgrGs and has a highly divergent C-terminal region (**Supplementary Figure S2** and **Supplementary Table S3**). Consistent with the VgrG comparisons, the DUF2169 and DUF4150 proteins with *vgrGa/c/d* also share 98–100% and 99–100% amino acid sequence identity, respectively (**Supplementary Table S3**). In contrast, VgrGb-associated DUF2169 and DUF4150 share only 26 and 35% sequence identities to those encoded in the other three *vgrG* loci respectively. The four predicted effector domains fused to DUF4150 or Rhs do not share high sequence similarity to each other or to known effectors, which could equip 1D1609 with diverse effector activities to fight with a wide range of competing bacteria. Homologs of *vgrGa*-, *vgrGb*-, *vgrGc*-, and *vgrGd*-associated effector genes are widely found in species belonging to Rhizobiales, suggesting that these are common effectors in this group.

Four VgrG Proteins Are Functionally Redundant in Mediating Hcp Secretion

In C58, VgrG1 and VgrG2 are functionally redundant in mediating Hcp secretion but specifically required for delivery of the cognate effectors Tde1 and Tde2, respectively (Lin et al., 2013; Bondage et al., 2016). Each of in-frame deletion mutants of *vgrGa*, *vgrGb*, *vgrGc* and *vgrGd* were generated to determine their functions in mediating Hcp secretion, a hallmark of a functional T6SS assembly. The Δ *tssL* mutant, with deletion of gene encoding the essential T6SS membrane component TssL, is used as a negative control. The secretion assay showed that all single *vgrG* deletion mutants remain active in Hcp secretion, suggesting that they are functionally redundant for assembly of a functional T6SS (**Figure 3A**). We are able to detect secretion of VgrG proteins from 1D1609 using polyclonal antibody against VgrG1 protein of C58, which can recognize all four VgrG proteins produced in 1D1609 and VgrG2 of C58. The Hcp and VgrG proteins are secreted in a T6SS-dependent manner because no Hcp and VgrG signals are detected in the secretion fraction of Δ *tssL* and RpoA (RNA polymerase α -subunit), a non-secreted protein, is not detectable in any secretion fraction. Hcp secretion remains active in double and triple *vgrG* deletion mutants but is only completely abolished when all four *vgrG* genes was deleted (**Figure 3B**). The overexpression of each of all four *vgrG* on a plasmid in the quadruple *vgrG* mutant restores Hcp secretion (**Figure 3C**). Furthermore, trans complementation of each 1D1609 *vgrG* constitutively expressed on a plasmid can also restore Hcp secretion in C58 double *vgrG* deletion mutant (Δ *vgrG1,2*) (**Figure 3D**). This is consistent with a previous study that either VgrG1 or VgrG2 is sufficient in mediating the secretion of Hcp in C58 (Lin et al., 2013). Indeed, all triple *vgrG* mutants wherein only one VgrG is present remains capable of mediating Hcp and VgrG secretion (**Figure 3E**). In Δ *vgrGabd* mutant, wherein only VgrGc is produced, Hcp secretion level is at lesser amount as compared to WT. The transcript expression

level of *vgrGc* is also low compared to the other *vgrGs* (Haryono et al., 2019). Altogether, our comprehensive secretion assays demonstrated that the four VgrGs are functionally redundant in mediating Hcp secretion. Single *vgrG* is sufficient for assembly of a functional T6SS although the degree of assembly efficiency may not be the same due to expression level or sequence divergence.

T6SS-Dependent Antibacterial Activity Against *Escherichia coli* Is Mainly Contributed by *vgrGa* and *vgrGd*

We previously showed that all tested *A. tumefaciens* strains including 1D1609 exhibit T6SS-mediated antibacterial activity against T6SS-negative *E. coli* (Wu et al., 2019b). Here, we further investigated the role of the four *vgrG* genes in interbacterial competition against *E. coli*. As a positive control, \sim 10x reduction of surviving *E. coli* target cells was observed when co-cultured with 1D1609 WT as compared to that of Δ *tssL*. However, each of single *vgrG* deletion mutants remains to have similar antibacterial activity to WT (**Figure 4A**). Further antibacterial activity assay of double, triple and quadruple *vgrG* mutants showed that the antibacterial activity of 1D1609 is completely lost in Δ *Gacd*, Δ *Gabd*, or Δ *Gabcd*, while Δ *Gbcd* and Δ *Gabc* triple *vgrG* mutants remain similar antibacterial activity to that of WT 1D1609 (**Figures 4B,C**). These results suggest that *vgrGa* and *vgrGd* alone is sufficient to contribute to the antibacterial activity of 1D1609. In contrast, *vgrGb* or *vgrGc* alone cannot exert any detectable antibacterial activity even though either *vgrG* alone (Δ *Gacd*, Δ *Gabd*) is sufficient in mediating Hcp and VgrG secretion (**Figure 3E**). However, when each single *vgrG* is constitutively overexpressed on a plasmid in quadruple *vgrG* mutant, *vgrGa*, *vgrGc*, and *vgrGd* but not *vgrGb* is able to partially restore antibacterial activity (**Figure 4D**). These results strongly suggest that endogenous *vgrGa* and *vgrGd* indeed play important roles for antibacterial activity while *vgrGb* plays no role in antibacterial activity to *E. coli*. The data that endogenous *vgrGc* plays no role but can exert its function in antibacterial activity when overexpressed are consistent with its low endogenous expression and high amino acid sequence identity to VgrGa and VgrGd.

The *vgrGa*- and *vgrGd*-Associated EI Pairs Are Responsible for Antibacterial Activity of 1D1609 and V2a Is a Nuclease of HNH/ENDO VII Superfamily

We also generated deletion mutants of each putative effector-immunity (EI) gene pairs individually and mutants with deletions in multiple putative EI pairs. Due to failure in generating the *vgrGc*-associated *v2c-v3c* EI pair deletion mutant after several attempts, we instead generated the deletion of the whole *vgrGc* cluster to assay the antibacterial effect of *vgrGc*-associated effector. Deletion of *vgrGa*- and *vgrGd*-associated EI pair partially compromised antibacterial activity against *E. coli* while deletion of *vgrGb*-associated EI pair or *vgrGc* cluster/associated toxin has no effect in compromising 1D1609's killing activity to *E. coli*, as compared to WT (**Figure 5A**). Importantly, any mutant minimally deleting both *vgrGa*- and *vgrGd*-associated EI pairs (Δ 2*EIad*, Δ 3*EIabd*, or Δ 4*EIabcd*) completely abolished the

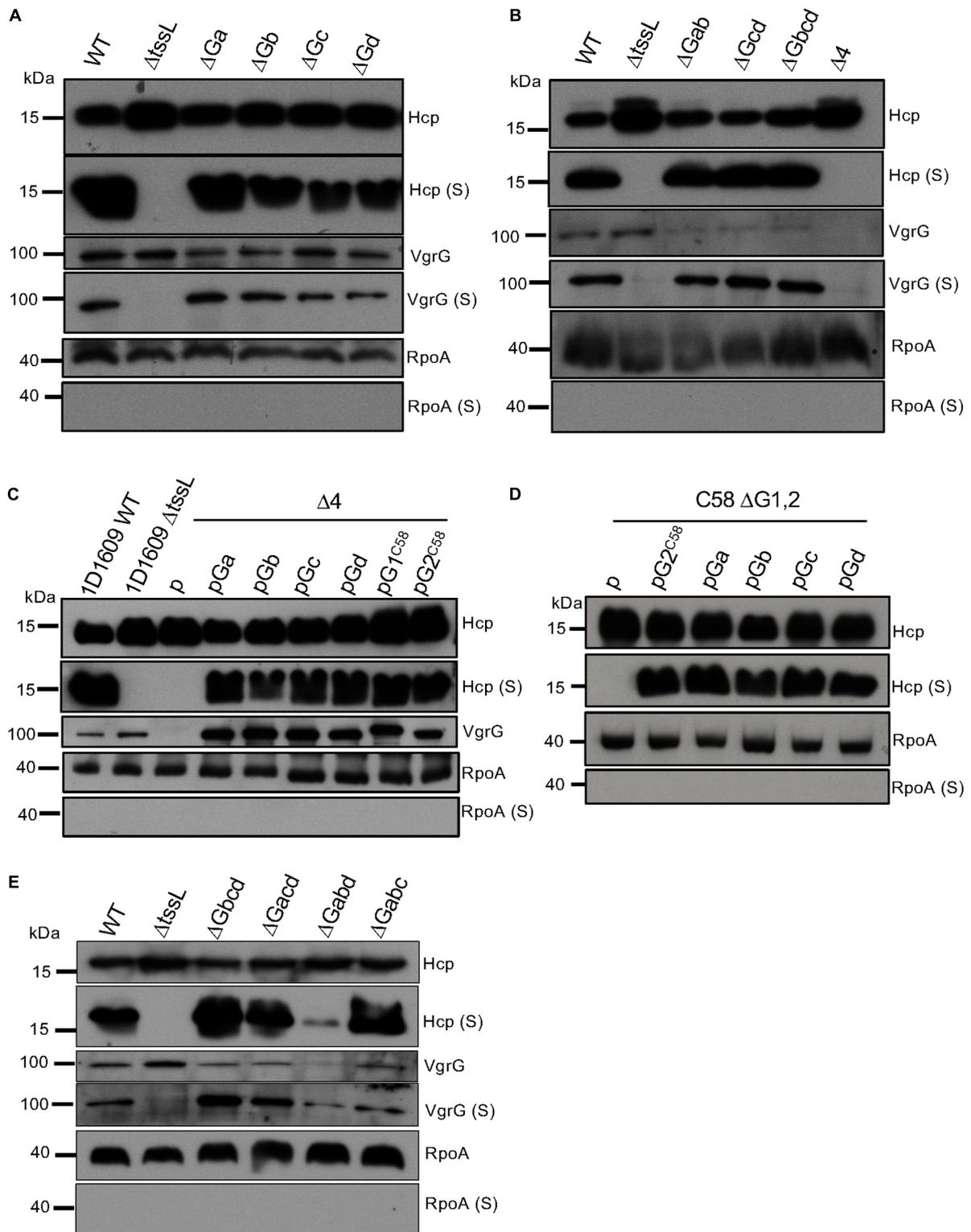
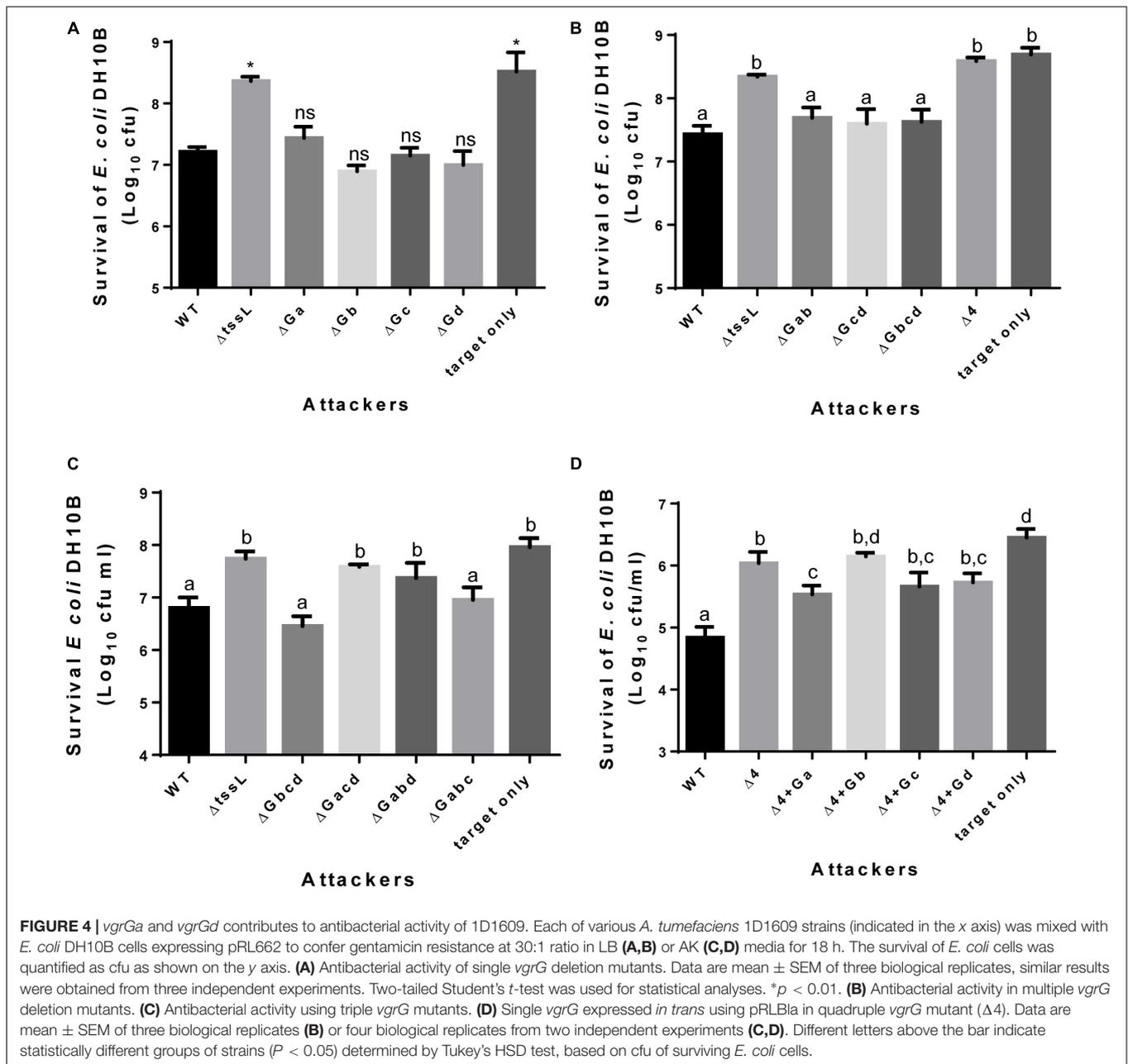


FIGURE 3 | The four VgrG proteins of 1D1609 are functionally redundant for Hcp secretion. Western blot analysis of cellular and secreted (S) proteins from single vgrG deletion mutants (A), multiple vgrG deletion mutants (B), overexpression of VgrG in 1D1609 quadruple vgrG mutant ($\Delta 4$) (C), and in C58 double vgrG deletion mutant ($\Delta G1,2$) (D), and triple vgrG deletion mutants (E). Various *A. tumefaciens* strains were cultured in LB (A,B,E) or AK (C,E) broth at 25°C and cellular and secreted (S) fractions were subjected to western blotting using anti-Hcp, anti-VgrG, and RpoA as indicated. The T6SS inactive mutant $\Delta tssL$ is a negative control and RpoA serves as a non-secreted protein control. Protein markers (in kDa) are indicated on the left. Similar results were obtained from 2–4 independent experiments.



antibacterial activity against *E. coli* (Figure 5B). These results suggest that *vgrGa*- and *vgrGd*-associated EI pairs contribute to antibacterial activity to *E. coli*.

Since single deletion of *vgrGa*-associated EI pair (ΔEIa) or in combination with additional EI-pair/s showed reduced or abolished antibacterial activity (Figures 5A,B), we considered *vgrGa*-associated effector V2a as the primary toxin and characterized its function. The putative effector gene *v2a* was expressed by an arabinose inducible promoter on plasmid pJN105 and its putative cognate immunity gene *v3a* was expressed constitutively on plasmid pTrc200 in *E. coli*. The results showed that the bacterial growth is inhibited when *v2a* is induced as compared to the vector control whereas co-expression with

v3a restores the growth (Figure 5C), indicating their toxin-immunity relationship.

V2a is 522-aa in length and contains a N-terminal PAAR-like DUF4150 domain and a C-terminal nuclease domain belonging to HNH/ENDOVII superfamily (pfam14412) of the treble cleft fold (Marchler-Bauer et al., 2017) (Figure 5D). This nuclease family has a conserved motif AHH, which is a predicted toxin module found in bacterial polymorphic toxin systems. In the AHH motif, the first His forms one of the catalytic metal-chelating ligands and the second His contributes to the active site that directs the water for phosphoester hydrolysis (Zhang et al., 2011). Partial sequence alignment of representative nuclease family of HNH/ENDO

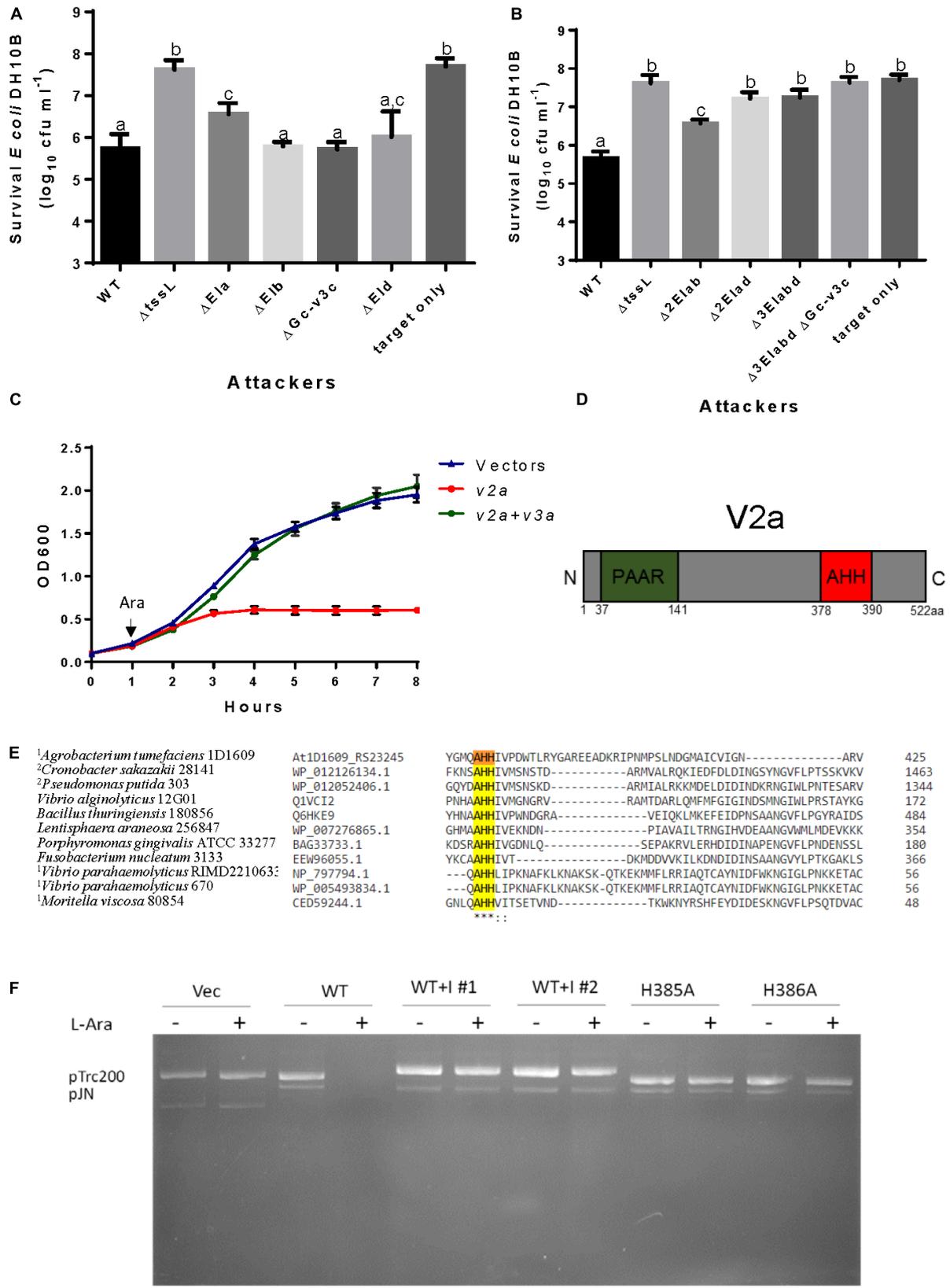


FIGURE 5 | Continued

FIGURE 5 | *vgrGa*- and *vgrGd*-associated EI pairs are responsible for antibacterial activity and V2a is a nuclease. **A.** *tumefaciens* 1D1609 strains (indicated in the x axis) was mixed with *E. coli* DH10B cells expressing pRL662 to confer Gentamicin resistance at 30:1 ratio in LB. The survival of *E. coli* cells was quantified as cfu as shown on the y axis when co-cultured with single **(A)** and multiple **(B)** EI pair deletion mutant. Data are mean \pm SEM of four biological repeats from two independent experiments. Different letters above the bar indicate statistically different groups of strains ($P < 0.05$) determined by Tukey's HSD test, based on cfu of surviving *E. coli* cells. **(C)** *E. coli* growth inhibition analysis in *vgrGa*-associated EI pair. *E. coli* cultures were induced with 1 mM IPTG at 0 h for the expression of putative immunity protein expressed on pTrc200 plasmid followed by L-arabinose (Ara) induction at 1 hr to induce putative toxin gene from pJN105 plasmid. Cell growth was recorded every hour at OD₆₀₀. Empty vectors were used as controls. Data are mean \pm SEM of three independent experiments. **(D)** Schematic diagram showing N-terminal PAAR region and C-terminal AHH nuclease domain of *vgrGa*-associated effector V2a. **(E)** Partial sequence alignment of representative nuclease family of HNH/ENDO VII superfamily with conserved AHH motif, modified from NCBI CDD sequence alignment of nuclease family of the HNH/ENDO VII superfamily with conserved AHH (pfam14412). The strain name and locus tag/accession number are on the left, and the conserved amino acid residues are indicated as * (identical) and: (similar) respectively. AHH motif is highlighted in yellow with the two His targeted for mutagenesis. AHH domain linked to DUF4150 domain¹ or Rhs² is indicated. **(F)** Nuclease activity assay. *E. coli* cells with pTrc200 and pJN105 plasmid (Vec) or derivatives expressing *v2a* (WT), WT with immunity protein (WT + I; #1 and #2 are two independent constructs) and catalytic site mutants (H385A, H386A) were induced with (+) or without (-) L-arabinose (L-Ara) for 2 h. Plasmid DNA was extracted and the degradation pattern was observed in agarose gel.

VII superfamily with conserved AHH motif is shown in **Figure 5E**.

To determine its nuclease activity, we examined the degradation of the plasmid DNA extracted from *E. coli* cells after *v2a* is induced by arabinose for 2 h. We showed that both pTrc200 and pJN-derived plasmids were not detectable from *v2a*-containing cells induced by arabinose, in contrast to the presence of both plasmids in vector control or *v2a*-containing cells not treated with arabinose (**Figure 5F**). The plasmid degradation is indeed caused by nuclease activity of V2a because the plasmid degradation is no longer detectable when the cells expressing the catalytic site (H385A and H386A) mutant or co-expression of *v3a* (**Figure 5F**). Taken together, we demonstrated that V2a is a T6SS effector that requires its AHH motif for nuclease activity. Moreover, this toxicity can be specifically neutralized by its cognate immunity protein. While this study was under way, a type VI nuclease effector Tse7 with GHH catalytic site (Tox-GHH2, pfam14412) from the same HNH/ENDO VII superfamily was reported in *P. aeruginosa* (Pissaridou et al., 2018). In conclusion, HNH nuclease superfamily appeared to be a widespread T6SS nuclease toxin family for antibacterial activity. These results strongly suggested the *v2a-v3a* toxin-immunity relationship, which supported their contribution to 1D1609's antibacterial activity to *E. coli*.

VgrG Can Be Exchanged Between C58 and 1D1609

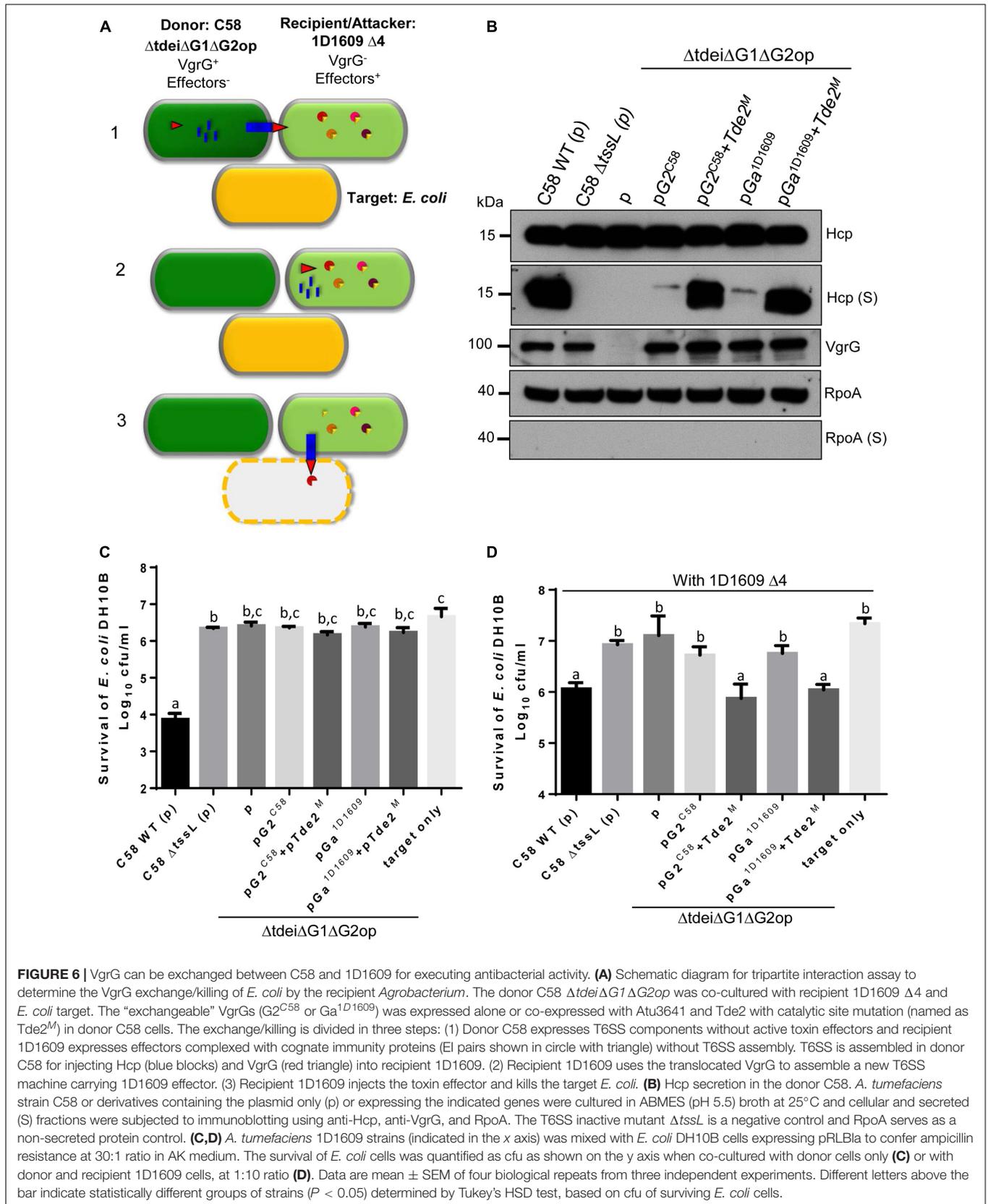
Considering that VgrGa, VgrGc and VgrGd of 1D1609 and VgrG2 of C58 are highly similar with identical C-terminal region and their DUF2169 and DUF4150 share high sequence similarity (**Supplementary Table S3**), it is plausible that these VgrG proteins can substitute each other's role in effector delivery. Previous work showed that Hcp and VgrG can be exchanged between *Vibrio cholerae* cells in a T6SS-dependent manner and can be reused to assemble a new T6SS (Vettiger and Basler, 2016). Thus, we hypothesized that *Agrobacterium* may take advantage of having multiple, highly similar VgrG to receive or donate this effector carrier from/to its siblings and the donated VgrG can be used by the recipient cell to kill another competing bacterial cell. To test this hypothesis, we designed a tripartite interbacterial transfer system that will allow us to see the killing of the target *E. coli* cell only when there is a functionally exchangeable VgrG

protein translocated from C58 to 1D1609. The nearly identical VgrGs (G2^{C58} and Ga^{1D1609}) expressed on a non-transferable pRL662 plasmid in C58 $\Delta tdei\Delta G1\Delta G2op$, a mutant deleting *vgrG1*, *tdei1*-*tdi1*, *vgrG2* operon, serves as a donor cell. 1D1609 $\Delta 4$ with deletion of all four *vgrG* genes but is still armed with effectors is designated as a recipient cell as well as an attacker to kill *E. coli* (**Figure 6A**). Since no antibacterial activity can be detected in C58 $\Delta tdei\Delta G1\Delta G2op$ (Ma et al., 2014) and 1D1609 $\Delta 4$ (**Figure 4B**), 1D1609 $\Delta 4$ can only exhibit antibacterial activity by obtaining VgrG proteins from C58 donor and using it for effector delivery to kill *E. coli*.

During this course of study, our group also discovered that Tde effector loading onto VgrG is critical for T6SS assembly and Hcp secretion (Wu et al., 2019a). Thus, aside from expressing G2^{C58} or Ga^{1D1609} in C58 $\Delta tdei\Delta G1\Delta G2op$ donor strain, a plasmid expressing Tde2 with catalytic site mutation and its cognate DUF2169 chaperone/adaptor (pTde2^M) was also included to ensure effective T6SS assembly and firing. Hcp secretion and antibacterial activity assays confirmed that the G2^{C58} or Ga^{1D1609} in C58 $\Delta tdei\Delta G1\Delta G2op$ donor strain remained active in Hcp secretion as WT level but completely lost the antibacterial activity against *E. coli* (**Figures 6B,C**). However, the donor-recipient-prey tripartite co-incubation showed that only the experimental set with C58 $\Delta tdei\Delta G1\Delta G2op$ donor strain harboring G2^{C58} or Ga^{1D1609} in the presence of pTde2^M can allow 1D1609 $\Delta 4$ to exhibit antibacterial activity to *E. coli* (**Figure 6D**). 1D1609 $\Delta 4$ co-cultured with donor strains C58 $\Delta tssL$, $\Delta tdei\Delta G1\Delta G2op$ harboring vector only, G2^{C58} or Ga^{1D1609} alone do not exhibit detectable killing activity to *E. coli*. These results suggest that 1D1609 can use the VgrG2^{C58} and VgrGa^{1D1609} delivered from C58 to kill *E. coli* and VgrG2^{C58} can function as an effector carrier in 1D1609.

DISCUSSION

Functional redundancy is a useful strategy for the pathogen to build a robust system (Galán, 2009). In this study, our phylogenomic analyses revealed that the majority of T6SS-harboring Proteobacteria encode multiple *vgrG* genes. Among them, *A. tumefaciens* strain 1D1609 is unique for having multiple VgrG paralogs (VgrGa, VgrGc, and VgrGd) with high sequence identity (99%). The three *vgrG* genetic modules have the



same gene orders encoding highly conserved VgrG-DUF2169-DUF4150. However the DUF4150-linked effectors do not share significant sequence similarities suggesting that these effectors may have distinct functions. This study provided genetic evidence that these nearly identical VgrG paralogs can carry not only their genetically linked cognate effector but also non-genetically linked effectors sharing the highly similar DUF4150. The functional replacement or exchange can occur for these proteins produced in the same bacterial cells or those taking from its isogenic sibling or close relatives during T6SS attacks. Thus, such functional redundancy may be a beneficial strategy for agrobacteria to compete in their ecological niche.

While several studies clearly demonstrated that VgrG is specifically required for delivery of cognate effector(s) encoded in the same *vgrG* genetic module (Hachani et al., 2014; Whitney et al., 2014) and C-terminal variable region of VgrG is the molecular determinant (Bondage et al., 2016; Flaugnatti et al., 2016), only few studies reveal the roles of highly homologous VgrG proteins in effector delivery. *D. dadantii* 3937 also encodes two highly similar VgrG homologs (VgrG_A and VgrG_B, **Supplementary Table S1**) which are both genetically linked to genes encoding homologous DUF1795-containing EagR chaperone and Rhs-linked effectors RhsA and RhsB (Koskiniemi et al., 2013). The homologous DUF1795 shares 97% amino acid sequence identity and RhsA and RhsB share 86% amino acid sequence identity with conserved N-terminal PAAR domain (99% identity). By sharing the highly similar chaperone and PAAR-Rhs regions, RhsA and RhsB effectors each harboring distinct C-terminal nuclease toxin domains could be delivered by not only its cognate but also non-cognate VgrG. Indeed, the competition assay revealed that either *vgrG_A* or *vgrG_B* is required for RhsB-mediated inhibition. The inhibitor cells without *vgrG_B* in $\Delta rhsA/rhsB^+$ background can still have competitive advantage. The advantage is lost when both *vgrG*s are deleted and restored when *vgrG_B* was expressed in double *vgrG* deletion mutant. Sharing the same VgrG carrier was also found in *Serratia marcescens* Db10, in which two different PAAR-domain containing Rhs effectors (Rhs1 and Rhs2) can functionally pair with the same VgrG protein (VgrG2) for delivery to target cells (Cianfanelli et al., 2016). The *rhs2* effector gene is not genetically linked to *vgrG2*, but both Rhs1 and Rhs2 have the same DUF1795 (EagR)-PAAR-Rhs gene organization. The EagR1 shares only 26% amino acid sequence identity with EagR2 and each specifically interacts with its cognate Rhs and is required for delivery (Alcoforado Diniz and Coulthurst, 2015; Cianfanelli et al., 2016). VgrG2 can form VgrG2-Rhs1-EagR1 or VgrG2-Rhs2-EagR2 complexes even though the N-terminal PAAR and full-length amino acid sequence of Rhs1 and Rhs2 share only 34 and 30% identity, respectively. In this case, high amino acid sequence identity in chaperone and PAAR is not required for functional exchange. However, they may share structural similarity that is sufficient for interacting with the same VgrG.

A recent study demonstrated that T6SS components, Hcp and VgrG, can be transferred and reused by *V. cholera* cells (Vettiger and Basler, 2016). An isogenic LacZ⁺ reporter strain was used to measure the level of LacZ released for detection of cell lysis when the isogenic donor cells translocate Hcp and

VgrG2 to isogenic recipient cells for T6SS assembly and firing. In our system, a tripartite interbacterial exchange/competition assay was designed to show evidence that highly similar VgrG proteins can be exchanged between two different *A. tumefaciens* genomospecies to kill the co-existing *E. coli*. Different *A. tumefaciens* genomospecies are known to co-exist in the same geographic area (Vogel et al., 2003). Several studies revealed that more than two genomospecies of *A. tumefaciens* can be found in the same crown gall or soil samples (Costechareyre et al., 2010; Bouri et al., 2016). Using tomato and maize seedlings, Gharsa et al. (2018) evaluated the competition of different *A. tumefaciens* genomospecies in soil and rhizosphere and concluded that related ecotypes can coexist. The initial competition alters the relative abundance, but does not eliminate the weaker strain. VgrG exchange may bring the advantages of sympatry and possessing different sets of T6SS gene clusters can maintain balance in sympatry. In *V. cholerae*, the bacterial strains that are incompatible (i.e., with different effector module sets), actively use their T6SS to kill susceptible strain during intra-species competition (Unterweger et al., 2014). However, in *A. tumefaciens* species, compatibility of T6SS EI pairs is not always predictive of competition outcomes (Wu et al., 2019b). C58 and 1D1609, which belongs to different genomospecies, can exhibit antibacterial activity against each other when the attacker is initiated with a higher relative abundance during an *in planta* interbacterial competition assay. However, C58 and 1D1609 can coexist with similar competitiveness when co-cultured at equal ratio despite their distinct EI pairs (**Supplementary Figure S4**). No significant difference of competitiveness (competitive index ~1) could be observed when C58 WT is co-inoculated with equal number of 1D1609 WT or $\Delta tssL$, or vice versa. This indicates that the two strains are proliferating equally *in planta* regardless of the presence or absence of a functional T6SS of *Agrobacterium* in different host plants. Interestingly, highly similar VgrGs are common in different *A. tumefaciens* genomospecies. In genomospecies 1 (G1), all (except 1D1108) share 100% identity while G8 (represented by C58 VgrG1 and two VgrGs in 1D132) share 95.7–99.8%. G7 (three VgrGs in 1D1609) and G8 (C58 VgrG2, 1D132 VgrG and three VgrGs in 1D1609) share 96.9–99.9% identity (**Supplementary Figure S3**). Thus, VgrG exchange might be common between different genomospecies, which may allow them to coexist and could in turn benefit *A. tumefaciens* sympatry in its ecological niche.

While not always evident by statistical analysis, deletion of single *vgrGa* ($\Delta vgrGa$) or its associated effector-immunity (ΔEIa), but not other single *vgrG* or *EI* pair mutants, showed reduction of antibacterial activity (**Figures 4A, 5A,B**). The data suggest that *vgrGa* genetic module play the primary role in antibacterial activity of 1D1609. The *vgrGa*-associated effector V2a belongs to different type of nuclease toxin distinct from the Tde1 and Tde2 nuclease toxins (toxin_43 domain with HxxD catalytic motif) identified in C58 (Ma et al., 2014). The use of enzymatic toxins such as nuclease is the most prominent theme in bacterial warfare (Galán, 2009). Using bioinformatics analysis, Ma et al. (2017) found that among more than 2,500 T6SS-dependent Rhs-CTs with N-terminal PAAR in 143 bacterial species, nuclease represents the major group. Among these,

the Rhs-AHH is the most dominant nuclease found in 66 bacterial species. Effectors with predicted AHH nuclease has been reported with antibacterial activity in *V. parahaemolyticus* VP1415 (Salomon et al., 2014) and *Pectobacterium carotovorum* subsp. *brasiliense* strain PBR 1692 (Bellieny-Rabelo et al., 2019) but no nuclease activity has been tested. In *Acinetobacter baylyi*, it was shown that the genomic DNA was completely degraded when Rhs2 with C-terminal AHH motif is expressed in *E. coli* (Fitzsimons et al., 2018). In this study, we characterize further to show that the AHH motif is indeed responsible for the DNase activity (Figure 5). The other conserved motif of HNH/ENDO VII superfamily nuclease has been reported in *P. putida* KT2440 Tke2 (Rhs-Tox-HNH) and Tke4 (Rhs-Tox-SHH) (Bernal et al., 2017) and functional characterization was done only in Tox-GHH2 in Tse7 in *P. aeruginosa* (Pissaridou et al., 2018). Among the 100 Soft-rot Enterobacteriaceae (SRE) genomes surveyed by Bellieny-Rabelo et al. (2019), about a quarter encodes AHH, suggesting that this nuclease is a common weapon deployed by both plant and animal pathogenic bacteria in interbacterial competition.

Among the four *vgrG* clusters encoded in 1D1609, *vgrGb* is distinct from the other three. Our data suggest that *vgrGb* and *v4b* encoding a putative toxin does not seem to play a role in antibacterial activity (Figures 4, 5A,B). This may be due to low expression level of this gene. Alternatively, V4b may target eukaryotic cells because it contains a nuclear localization signal and has an extensive structural similarity to the insecticidal TcdB2-TccC3 toxin of *Photorhabdus luminescens* and YenC2 toxin of *Yersinia entomophaga* (Supplementary Table S2). The activity of the remaining effectors remains unknown and is currently under investigation.

In conclusion, *Agrobacterium* has developed a flexible mode of T6SS effector delivery, by using highly similar VgrGs, either produced endogenously or injected from its close relatives, for T6SS assembly and firing. VgrG and PAAR are among the least abundant T6SS components (Lin et al., 2019) and limiting VgrG can result in reduced number of T6SS assemblies (Vettiger and Basler, 2016). Therefore, it is plausible that having multiple highly similar VgrGs may enhance robustness and abundance of T6SS assemblies. This flexibility could allow them to be an ally to its sister cells and an effective weapon against distantly related bacterial competitors. The knowledge gained in this study can help advance the understanding of mechanisms and physiological roles of multiple VgrG proteins and associated effectors and may provide new insights for sympatric speciation.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains, plasmids and primers used in this study are listed in Supplementary Tables S4, S5. *A. tumefaciens* strains were grown in 523 medium at 25°C while *E. coli* strains were grown in LB medium at 37°C. In-frame deletion mutants in *Agrobacterium* were generated using a suicide plasmid via double crossing over by electroporation or by conjugation (Ma et al., 2009). The mutants were confirmed by colony

PCR and/or Southern blot analysis (Supplementary Figure S5). Site directed mutagenesis was done using overlapping PCR (Ho et al., 1989).

Phylogenetic and Sequence Analysis

All bioinformatics tools were used with the default parameters unless stated otherwise. The function of putative effectors were inferred from NCBI's Conserved Domain Database (CDD) based on BLASTP searches (Boratyn et al., 2013; Marchler-Bauer et al., 2017) and Protein Homology/analogy Recognition Engine (Phyre2) (Kelley et al., 2015). SignalP was used to predict signal peptides (Petersen et al., 2011). PSORTb version 3.0.2 was used to predict sub-cellular localization of proteins (Yu et al., 2010).

For the phylogenomic analysis to investigate the VgrG diversity among Proteobacteria, the VgrG homologs from five representatives (i.e., *A. tumefaciens* 1D1609, *Burkholderia thailandensis* E264, *Geobacter sulfurreducens* PCA, *Helicobacter cinaedi* CCUG 18818, and *P. aeruginosa* PAO1) were used as queries to run BLASTP searches against the NCBI non-redundant protein database (*e*-value cutoff = 1e-15, max target sequences = 100,000). Spurious hits, defined as hits with the high scoring pairs (HSP) accounting for <74% of the query sequence or sequence similarity <48% within the HSP, were removed. The resulting lists from different query species were combined to remove redundant hits. The combined list was further manually curated to keep only the targets with complete genome sequences available in GenBank. Based on the taxonomy information of the targets, the list was iteratively trimmed to achieve a balance taxon sampling with ~50 genomes to represent all major lineages within Proteobacteria. One Planctomycetes, *Pirellula staleyii* DSM 6068, was included as the outgroup to root the species phylogeny (Figure 1). In addition to the aforementioned data set for phylum-level (i.e., Proteobacteria) diversity, a second data set containing 11 *Agrobacterium* genomes (Wu et al., 2019b) was compiled to examine the genus-level VgrG diversity; *Neorhizobium galegae* HAMBI 540 was included as the outgroup for this second data set (Supplementary Figure S3).

After genome selection, the procedures for homologous gene identification and molecular phylogenetic analysis were based on those described in our previous studies (Lo et al., 2013; Lo and Kuo, 2017). Briefly, the homologous genes among all genomes were identified by using OrthoMCL (Li et al., 2003). The single-copy genes shared by all genomes were used for inferring the species phylogeny. Additionally, all *vgrG* homologs in these genomes, defined as the genes containing at least 80% of the VgrG domain (TIGR03361), were extracted for inferring the gene tree. The protein sequences were aligned using MUSCLE (Edgar, 2004) and the molecular phylogenies were inferred using MrBayes (Ronquist and Huelsenbeck, 2003). The amino acid substitution model was set to mix with gamma-distributed rate variation across sites and a proportion of invariable sites. The number of rate categories for the gamma distribution was set to four. The Markov chain Monte Carlo analysis was set to run for 1,000,000 generations and sampled every 100 generations. The first 25% of the samples were discarded as the burn-in. Furthermore, the protein sequence identity among all VgrG homologs were calculated using PROTDIST (Felsenstein, 1989). The amino

acid sequence identity and similarity percentage of VgrG and VgrG-associated proteins were determined using Vector NTI Advance 11.5.4.

Secretion Assay

Secretion assay from liquid culture of *A. tumefaciens* grown in either LB rich medium or AB-MES minimal medium was done as previously described (Ma et al., 2009). Proteins from cellular and secreted fractions were resolved by SDS-PAGE and transferred onto a PVDF membrane by using a transfer apparatus (Bio-Rad). The membrane was probed with primary antibody against C58 VgrG1 (1:1,000), which recognizes the C58 VgrG2 and all four VgrGs of 1D1609, Hcp (1:2,500), and RpoA (1:7,500) (Lin et al., 2013), followed by incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:30,000) and visualized with the ECL system (PerkinElmer).

Interbacterial Competition Assay

Escherichia coli killing assay was performed as described previously (Bondage et al., 2016). In brief, overnight culture of *A. tumefaciens* and *E. coli* strain were adjusted to OD₆₀₀ 0.1 and incubated at 25°C for 4 h. *A. tumefaciens* and *E. coli* cells were mixed at a 30:1 ratio (OD 0.3:0.01) and spotted on LB agar (1.5%) plates. After 18 hr incubation at 25°C, the co-cultured cells were collected, serially diluted and plated on LB agar containing appropriate antibiotics to quantify surviving *E. coli* by counting colony forming unit (cfu). When enhanced killing activity is desired, an optimized growth medium, named as *Agrobacterium* Kill-triggering, AK medium (3 g K₂HPO₄, 1 g NaH₂PO₄, 1 g NH₄Cl, 0.15 g KCl, 9.76 g MES in 900 mL ddH₂O, pH5.5) modified from AB-MES medium and solidified by 2% (w/v) agar, was used instead. Data are expressed as mean ± SEM (standard error of the mean) from three independent experiments. Statistics was done using one-way ANOVA and Tukey's honestly significance difference (HSD) test¹.

In planta bacterial competition assay was performed as described previously (Ma et al., 2014). *A. tumefaciens* strain C58 transformed with gentamicin resistance-conferring pRL662 plasmid and strain 1D1609 conferring spectinomycin resistance were mixed at 1:1 (OD 1) ratio and infiltrated into 6- to 7-week-old *Nicotiana benthamiana* and *Medicago truncatula* leaves. The competition outcome was quantified at 0 h and 24 h by counting cfu on LB plates with appropriate antibiotics. 1D1609/C58 ratio at *t* = 24 h was divided by 1D1609/C58 ratio at *t* = 0h to calculate the competitive index.

Growth Inhibition Assay

Growth inhibition assay was performed as described previously (Ma et al., 2014). In brief, overnight cultures of *E. coli* DH10B strain with vectors or their derivatives were adjusted to OD₆₀₀ 0.1. The expression of the tested immunity protein was induced by 1 mM IPTG for 1 h before L-arabinose (0.2% final concentration) was added to induce expression of the toxin.

Plasmid DNA Degradation Analysis in *E. coli* Cells

In vivo plasmid DNA degradation analysis was performed as described previously (Ma et al., 2014). In brief, overnight cultures of *E. coli* DH10B strain with empty vectors or derivatives expressing *v2a* and catalytic site mutant were harvested and adjusted to OD₆₀₀ 0.3. L-arabinose (0.2%, final concentration) was added to induce the expression and cultured for 2 h. Equal amounts of cells were used for plasmid DNA extraction and equal volume of extracted DNA was resolved in agarose gel followed by ethidium bromide staining.

Southern Blot Hybridization

Southern blot hybridization was performed as described (Sambrook, 2001). In brief, genomic DNA (gDNA) was extracted from overnight cultures of selected strains using Genomic DNA purification Kit (Promega) as per manufacturer's instructions. About 35 µg of gDNA was digested using NEB restriction enzymes (NsiI and PvuII) and resolved in 0.8% agarose gel, 50 V, 3 h. The DNA fragments were transferred to a positively charged nylon membrane (Roche Diagnostics). Nylon membranes were cross-linked and used for hybridization with DIG-labeled probe (Roche). Hybridization was done overnight at 65°C using hybridization solution (FastHyb-Hybridization solution, BioChain). The washing, blocking and detection were done using Roche Wash and Block Buffer Set and DIG DNA Labeling and Detection Kit, according to manufacturer's instructions (Roche). The membrane was exposed to X-ray for detection. The probe for the four *vgrGs* (*vgrGa*, *vgrGb*, *vgrGc*, and *vgrGd*) and putative toxin genes (*v2a*, *v2c*, *v2d*, and *v4b*) was prepared using the plasmid DNA *vgrGa*-pJN105, *v2d*-pJN105, and *v4b*-pJN105, respectively. The primer sets used are listed in **Supplementary Table S5**.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

E-ML and MS conceptualized the study. MS, S-TC, and C-JC worked on the data curation. E-ML, C-HK, C-FW, and MS worked on the methodology. C-HK, S-TC, MS, and C-JC carried out the investigation. E-ML was responsible for project administration and resources. E-ML and C-HK supervised the study. MS, C-HK, and E-ML wrote the manuscript. All authors reviewed and approved the manuscript.

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¹http://astatsa.com/OneWay_Anova_with_TukeyHSD/

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.03004/full#supplementary-material>

FIGURE S1 | Bayesian phylogeny of the VgrG homologs identified in the representative Proteobacteria genomes presented in **Figure 1**. The color coding is based on the class: Alphaproteobacteria (black), Betaproteobacteria (blue), Deltaproteobacteria (cyan), Epsilonproteobacteria (purple) and Gammaproteobacteria (green). Numbers on the branches indicate the support levels based on posterior probabilities, only values > 60% were shown.

FIGURE S2 | Multiple sequence alignment of VgrG amino acid sequences of 1D1609 and C58. All VgrG homologs were aligned and conserved amino acid residues are shaded in yellow and variable residues are in blue or green, while the C-terminal extension of C58 VgrG1 not homologous to the other VgrGs is unshaded. The red and blue lines indicate the predicted gp27 and gp5 domains, respectively. The amino acid residue number is indicated.

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FIGURE S3 | VgrG tree of the 11 *A. tumefaciens* strains. All VgrG homologs in these 11 genomes were extracted for inferring the gene tree. The VgrG orthologs share $\geq 95\%$ identity in amino acid sequence were highlighted. The genospecies and strain name for each VgrG indicated by a locus tag are shown.

FIGURE S4 | *In planta* competition experiments between C58 and 1D1609 at 1:1 ratio. *A. tumefaciens* strain C58 harboring gentamicin resistance-conferring pRL662 and strain 1D1609 which was selected in spectinomycin plate were mixed at 1:1 ratio and infiltrated into leaves of 6- to 7-week-old *Nicotiana benthamiana* (A) and *Medicago truncatula* (B). The competition outcome was quantified at 0 and 24 h post-infection by counting cfu on LB plates with appropriate antibiotics. Data are mean \pm SEM, with each data point indicating the competitive index of 4–6 biological replicates from a total of 2–3 independent experiments. No statistical difference ($P > 0.05$) could be detected among different samples as determined by Tukey's HSD test.

FIGURE S5 | Southern blot analysis of mutants generated in this study. Schematic diagram showing probes used for Southern blotting, restriction enzyme cleavage sites and expected sizes. The genomic DNA is hybridized with (A) 821-bp vgrG probe with homology to all four vgrG genes (*vgrGabcd*) was used to confirm vgrG mutants; (B) 478-bp v2 probe with homology to *v2acd* was used to confirm *v2a*, *v2c*, and *v2d* mutant and (C) 778-bp *v4b* probe was used to confirm *v4b* mutant. Each lane contains 30 μ g of genomic DNA digested with *NsiI* only or combined with *PvuII*. The expected size (in bp) is indicated on the side of the blot.

TABLE S1 | Percentage identity of VgrG within genomes with highly identical multiple VgrGs. The locus tag of each homolog is shown in rows and column and highlight shows $\geq 95\%$ identity.

TABLE S2 | Predicted functions/domains of toxin/s associated with each vgrG based on Phyre or NCBI CDD search and BLASTP.

TABLE S3 | Amino acid sequence similarity/identity among the protein homologs encoded in the vgrG genetic modules in *A. tumefaciens* strains C58 and 1D1609.

TABLE S4 | Bacterial strains and plasmids.

TABLE S5 | Primers used in this study.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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