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*e*-Xtra*

# Plant-Pathogenic *Agrobacterium tumefaciens* Strains Have Diverse Type VI Effector-Immunity Pairs and Vary in In-Planta Competitiveness

**Chih-Feng Wu,[1,2] Mary Nia M. Santos,[1] Shu-Ting Cho,[1] Hsing-Hua Chang,[1] Yi-Ming Tsai,[1] Delaney A. Smith,[2] Chih-Horng Kuo,[1,†] Jeff H. Chang,[2,3,†] and Erh-Min Lai[1,†]**

[1] Institute of Plant and Microbial Biology, Academia Sinica, Taipei 11529, Taiwan
[2] Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR, U.S.A.
[3] Center for Genome Research and Biocomputing, Oregon State University

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**The type VI secretion system (T6SS) is used by gram-negative bacteria to translocate effectors that can antagonize other bacterial cells. Models predict the variation in collections of effector and cognate immunity genes determine competitiveness and can affect the dynamics of populations and communities of bacteria. However, the outcomes of competition cannot be entirely explained by compatibility of effector-immunity (EI) pairs. Here, we characterized the diversity of T6SS loci of plant-pathogenic *Agrobacterium tumefaciens* and showed that factors other than EI pairs can impact interbacterial competition. All examined strains encode T6SS active in secretion and antagonism against *Escherichia coli*. The spectra of EI pairs as well as compositions of gene neighborhoods are diverse. Almost 30 in-planta competitions were tested between different genotypes of *A. tumefaciens*. Fifteen competitions between members of different species-level groups resulted in T6SS-dependent suppression in in-planta growth of prey genotypes. In contrast, ten competitions between members within species-level groups resulted in no significant effect on the growth of prey genotypes. One strain was an exceptional case and, despite encoding a functional T6SS and toxic effector protein, could not compromise the growth of the four tested prey genotypes. The data suggest T6SS-associated EI pairs can influence the competitiveness of strains of *A. tumefaciens*, but genetic features have a significant role on the efficacy of interbacterial antagonism.**

†Corresponding authors: for queries regarding genome sequences, C.-H. Kuo; chk@gate.sinica.edu.tw; for queries regarding T6SS-mediated competition, J. H. Chang; changj@science.oregonstate.edu or E.-M. Lai; emlai@gate.sinica.edu.tw

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Bacteria employ many strategies to compete for habitable space and nutrients. In exploitative competition, bacteria can alter metabolic strategies to change the external environment, produce public goods to compete for nutrients, or hasten colonization to gain access to new niches (Ghoul and Mitri 2016). In interference competition, bacteria can secrete products such as antibiotics and bacteriocins to reduce the fitness of competitors (Hibbing et al. 2010). These competitive activities influence the establishment and maintenance of structures of microbial communities and, in turn, the functions provided to hosts (Nadell et al. 2016).

Some bacteria employ specialized secretion systems to deliver toxic effector proteins into competing cells (Chang et al. 2014). The type VI secretion system (T6SS) is one such specialized apparatus. The T6SS resembles a membrane-associated, inverted phage tail-like structure (Cianfanelli et al. 2016b). It is a versatile apparatus that can be deployed for virulence toward eukaryotes, to mediate horizontal gene transfer, or acquire nutrients from the environment (Borgeaud et al. 2015; Chen et al. 2016; Ma and Mekalanos 2010; Si et al. 2017; Wang et al. 2015). The most frequently observed use of T6SS is in contact-dependent antagonism of bacterial cells (Russell et al. 2014). Fitness of prey cells is demonstrably compromised by T6SS-using strains when cocultured and coinfected in hosts (Bernal et al. 2017; Fu et al. 2013, 2018; Ma et al. 2014; Sana et al. 2016; Wexler et al. 2016).

Within the Proteobacteria phylum, T6SSs are defined on the basis of 13 core type six subunit (Tss) A-M proteins (Boyer et al. 2009). These assemble into substructures, which are a cell envelope–spanning complex, a baseplate complex, and the contractile tail–like substructure (Basler 2015). The latter substructure consists of a TssBC outer sheath, a TssD (Hcp) tube-like structure, and a spike-like structure formed by TssI (VgrG) and PAAR (proline-alanine-alanine-arginine). VgrG is homologous to gp27 and gp5, the puncturing device of T4 bacteriophage (Pukatzki et al. 2007). PAAR proteins are hypothesized to enhance the functionality of the spike-like structure (Shneider et al. 2013). It was recently suggested that PAAR-encoding genes should also be considered core to T6SS, as they are present in all T6SS-encoding loci and PAAR is necessary for function (Burkinshaw et al. 2018; Cianfanelli

et al. 2016a). Upon contraction of an outer sheath, the inner Hcp tube and VgrG are propelled outward, launching effectors that are either packed into the Hcp tube or associated to VgrG (Shneider et al. 2013; Silverman et al. 2012).

Effectors deployed for interbacterial competition generally target the cell wall, inner membrane, or nucleic acids of target cells, though effectors with different targets have been reported (Lien and Lai 2017). The genes of these effectors are always linked to a cognate immunity protein–encoding gene and present as modules referred to as effector-immunity (EI) pairs. The immunity proteins function to protect the T6SS-employing cells from self-intoxication. In addition, the vertical inheritance of immunity genes leads to protection from attack by kin, and EI pairs may, thus, confer kin recognition and promote clustering and social behaviors of bacteria (Wenren et al. 2013). However, EI pairs can also be horizontally acquired, occurrences of which are predicted to cause changes in population and community structures (Thomas et al. 2017).

Agrobacteria are best known for their ability to genetically transform plants and cause crown galls or hairy roots (Nester 2015). These bacteria are broad and narrow host–range pathogens that form a polyphyletic group (Costechareyre et al. 2010). Their phylogeny and taxonomic classifications have not been resolved and multiple schemes are currently used. The focal group of this study is *Agrobacterium tumefaciens* (also known as biovar 1). This group circumscribes multiple clusters called genomospecies, which evidence suggests are each representative of species (Lassalle et al. 2011). Genomospecies G8 for example, has been assigned *Agrobacterium fabrum.* We will continue to use the *A. tumefaciens* binomial term and genomospecies in reference to the clade and the species-level groups circumscribed by *A. tumefaciens*, respectively.

*A. tumefaciens* can persist in soil and in association with plants. During the transition from soil to a host environment, there are significant changes in the expression of many traits. During growth in planta or in the rhizosphere, which is mimicked by growing *A. tumefaciens* in acidic conditions (pH = 5.5), features associated with a motile, individual lifestyle are downregulated while those associated with a sessile, social lifestyle are upregulated (Barton et al. 2018). The latter is associated with the induced expression of the T6SS (Heckel et al. 2014; Wu et al. 2012; Yuan et al. 2008). We previously showed that in planta, *A. tumefaciens* C58 can antagonize a fully susceptible mutant of *A. tumefaciens* lacking all three EI pairs (Ma et al. 2014). In contrast, in acidic media, though the T6SS is active and *A. tumefaciens* C58 can antagonize *E. coli, A. tumefaciens* C58 failed to antagonize the mutant strain (Ma et al. 2014; Wu et al. 2018). These observations suggest the host environment influences the efficacy of T6SS-mediated antagonism.

In *A. tumefaciens*, the core proteins are encoded in two adjacent and divergently expressing operons. The *imp* locus encodes most of the core proteins and those involved in the posttranslational regulation of the T6SS (Lin et al. 2013, 2014, 2018). One core protein is TssL, an essential protein of the inner membrane complex (Ma et al. 2012). In strain C58, TssL is also a phosphoprotein necessary for posttranslational regulation of the T6SS (Lin et al. 2014). The *hcp* locus encodes the ClpV ATPase, Hcp, VgrG, a protein that functions as one or both an adaptor and chaperone (adaptor/chaperone) and a PAAR protein. In addition, the *hcp* locus of strain C58 has two EI pairs. One EI pair includes Tae, a putative peptidoglycan amidase, and the other includes Tde1, a DNase (Ma et al. 2014). Strain C58 also has an accessory *vgrG* locus distal to *imp* and *hcp*. This *vgrG2* locus includes genes that encode an adaptor and an EI pair. The effector Tde2 (Atu3640) is also a DNase and includes a DUF4150 domain analogous to PAAR. The carboxy-terminal portions of VgrG confer specificity to an adaptor protein and DNase effector (Bondage et al. 2016).

Our goal was to test the impact of genetic variation of *A. tumefaciens* on in-planta competition. We characterized the T6SSs and EI pairs of members of different species-level groups of *A. tumefaciens.* In all examined strains, an *imp* locus is present and conserved in sequence as well as structure. We confirmed the functionality of the T6SS for tested strains. Regions downstream of *vgrG* in *hcp* loci and accessory *vgrG* loci varied in gene order and composition, suggesting that strains vary in their spectra of EI pairs. Strains exhibited significant antagonistic activities when paired in planta against genetically distant competitors. The one exception to observations was strain 12D1. Its T6SS is demonstrably functional and its effector is toxic when directly expressed in *E. coli*. Yet, 12D1 exhibited no measurable T6SS-dependent effect when competed against strains predicted to be susceptible. Within species-level groups, strains, despite having different EI pairs, showed little to no fitness effect on competitors.

## RESULTS

### Genome characteristics and genetic divergence of *A. tumefaciens*.

A total of seven newly finished genome sequences for *A. tumefaciens* (biovar 1) strains were determined and compared with those of C58 (genomospecies G8), Ach5 (G1), 1D1609 (G7), and H13-3 (G1) (Table 1) (Cho et al. 2018; Goodner et al. 2001; Huang et al. 2015; Slater et al. 2013; Wibberg et al. 2011; Wood et al. 2001). All 11 strains share the same chromosomal organization of having one circular chromosome and one linear chromid, which is distinct from those found in *Agrobacterium rhizogenes* (biovar 2) and *Agrobacterium vitis* (biovar 3) (Slater et al. 2013, 2009). The presence of two signature plasmids, pAt and an oncogenic tumor-inducing (Ti) plasmid, in *A. tumefaciens* is variable. For example, 12D1 lacks a pAt plasmid, while H13-3 and 12D13 both lack oncogenic plasmids.

Genome-scale analysis inferred a strongly supported molecular phylogeny that is consistent with previous results based on *recA* typing for genomospecies designation (Fig. 1A) (Costechareyre et al. 2010, 2009). More importantly, examination of gene content revealed that strains belonging to the same genomospecies mostly shared >85% of their gene content, while those belonging to different genomospecies usually shared approximately 80% of the gene content (Fig. 1B). This discontinuity in the level of gene content similarity suggested that genomospecies are likely to be true ecological species, as proposed previously (Lassalle et al. 2011). Strain 12D13 is exceptional in being more divergent with respect to gene content, but it maintains high levels of sequence similarity among homologous genes. Finally, all strains are highly diverged, with ≤60% of shared gene content from strain K84 of *A. rhizogenes.*

The addition of genome sequences from members of genomospecies G1, G4, and G8 increased the sampling depth within *A. tumefaciens* as well as its genomospecies and provided the foundation to study the effect of variation of EI pairs on interbacterial competition. The translated sequences of genes from the *imp* and *hcp* operons of strain C58 were used as queries in searches (Fig. 1C). All 11 genome sequences have an *imp* locus identical in composition as well as order as the *imp* locus of strain C58 (Lin et al. 2013). Likewise, the upstream portions of the *hcp* loci are also conserved, as all strains had homologs of *tssH* (*clpV*), *tssD* (*hcp*), *tssI* (*vgrG*), and the *tae-tai* EI pair. In all strains, the *imp* and *hcp* loci are located on the linear chromid.

**Table 1.** Genome characteristics of *Agrobacterium tumefaciens* strains[a]

| Genomospecies | Strain | Accession | Size (bp) | Number of CDSs | Number of rRNAs | Number of tRNAs | Number of Plasmids | Ti | Source/reference |
|---|---|---|---|---|---|---|---|---|---|
| G8 | C58 | AE007869 to AE007872 | 5,674,258 | 5,355 | 12 | 56 | 2 | + | Goodner et al. 2001; Wood et al. 2001 |
| G8 | 1D132 | CP033022 to CP033026 | 5,553,114 | 5,148 | 12 | 54 | 3 | + | Wroblewski et al. 2005 |
| G8 | 12D13 | CP033034 to CP033038 | 5,416,317 | 5,055 | 12 | 53 | 3 | – | C. I. Kado |
| G7 | 1D1609 | CP026924 to CP026928 | 5,985,137 | 5,630 | 12 | 53 | 3 | + | Cho et al. 2018 |
| G4 | 1D1460 | CP032926 to CP032929 | 5,683,743 | 5,387 | 12 | 54 | 2 | + | Wroblewski et al. 2005 |
| G4 | 12D1 | CP033031 to CP033033 | 5,446,032 | 5,047 | 12 | 53 | 1 | + | C. I. Kado |
| G1 | 1D1108 | CP032921 to CP032925 | 5,767,533 | 5,359 | 15 | 57 | 3 | + | Wroblewski et al. 2005 |
| G1 | 15955 | CP032917 to CP032920 | 5,870,997 | 5,458 | 15 | 56 | 2 | + | S. B. Gelvin |
| G1 | A6 | CP033027 to CP033030 | 5,939,134 | 5,527 | 15 | 56 | 2 | + | S. B. Gelvin |
| G1 | Ach5 | CP011246 to CP011249 | 5,668,655 | 5,276 | 15 | 56 | 2 | + | Huang et al. 2015 |
| G1 | H13-3 | CP002248 to CP002250 | 5,573,770 | 5,345 | 15 | 57 | 1 | – | Wibberg et al. 2011 |
| *Agrobacterium rhizogenes* | K84 | CP000628 to CP000632 | 7,273,300 | 6,684 | 9 | 51 | 3 | – | Slater et al. 2009 |

[a] CDSs = coding sequences; Ti = tumor-inducing.

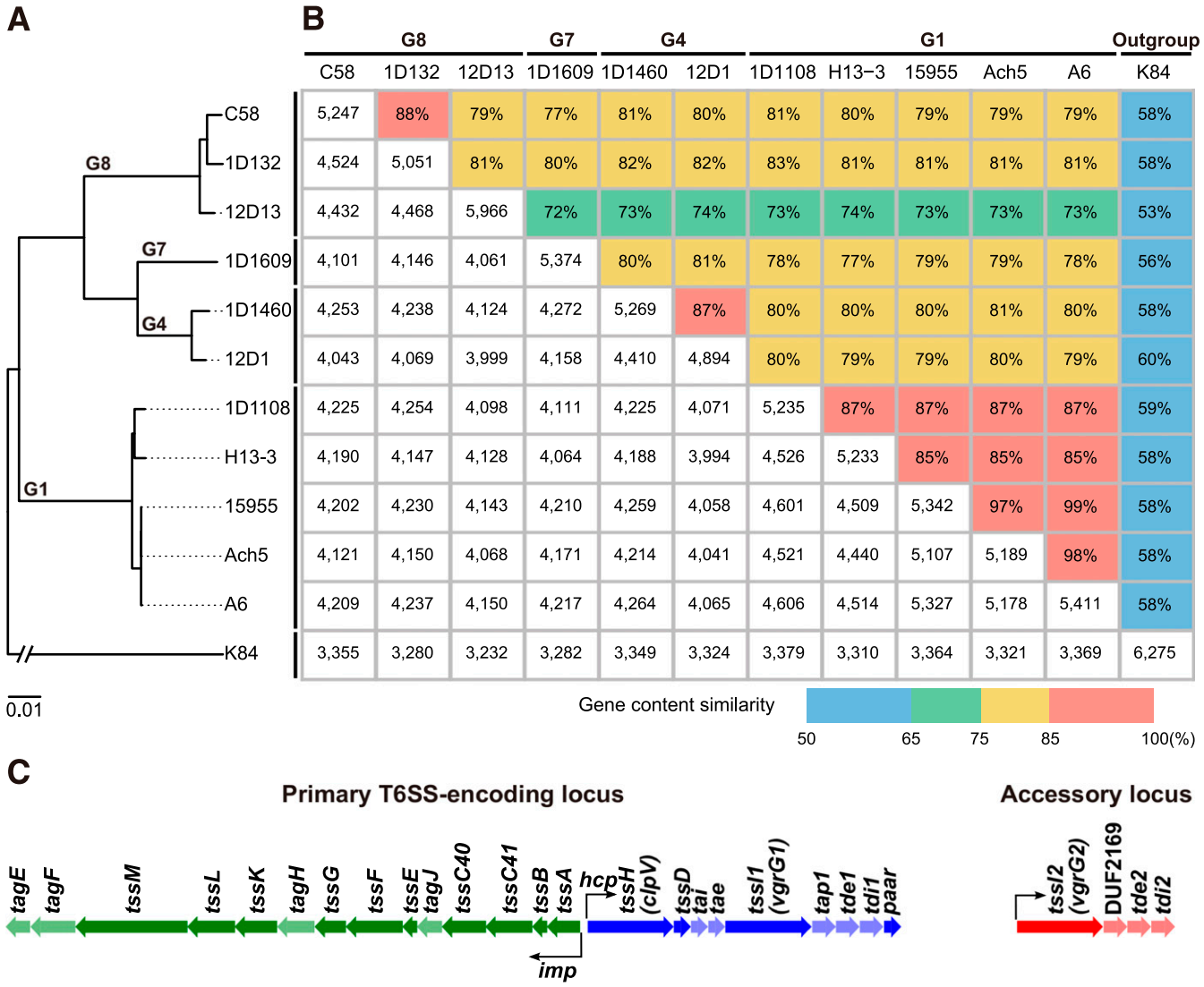


**Fig. 1.** Genomic divergence among representative *Agrobacterium* strains. **A,** Maximum likelihood phylogeny based on the concatenated alignment of 2,696 single-copy genes shared by all strains (909,997 aligned amino acid sites). All internal nodes received >93% bootstrap support. K84 is a strain of *A. rhizogenes* included as the outgroup to root the tree. **B,** Gene content comparison. Diagonal: number of homologous gene clusters identified within each genome; below diagonal: number of homologous gene clusters shared by each genome-pair; above diagonal: percent of genes shared by each pair of genomes (i.e., number of gene clusters shared divided by the average number of gene clusters between two genomes). **C,** Diagram of the type VI secretion system (T6SS)-associated loci of C58. The *imp* operon is green, the *hcp* operon is blue, and the *vgrG2* accessory locus is red. Opaque symbols represent genes core to the T6SS.

**Tested strains have functional T6SS that vary in in-vitro activity against *E. coli*.**

A subset of the strains were inoculated into an acidic minimal medium that induces expression to test whether the T6SSs are functional (Wu et al. 2012). We did not include strains Ach5 and H13-3 of genomospecies G1 because their T6SS-associated loci are predicted to be identical to those of strains 15955 and A6. Hcp and Tae were detected in the media fraction of each strain. Hcp is a commonly used marker for T6SS secretion, while Tae is an effector conserved in each of the strains of *A. tumefaciens* (Fig. 2A) (Lin et al. 2013). Tde1 was detected in only strains C58 and 1D132 of genomospecies G8 (Fig. 2B). RpoA, a DNA-dependent RNA polymerase was not detected in media fractions, suggesting no substantial leakage from cellular fractions. We also generated and tested Δ*tssL* mutants for most of the strains. Hcp was not detected in the media fractions of any of the Δ*tssL* mutants but could be detected in the media fractions of mutant strains complemented with their cognate *tssL* gene (Fig. 2C). These data indicate that all the tested strains encode functional T6SS. We could not isolate a mutant for strain 12D13, and this strain was not carried forward in subsequent experiments.

As another test of T6SS activity, the seven genotypes were competed against *E. coli* K12 (pRL662) at a 30:1 (predator/prey) ratio on agar plates (Fig. 3) (Vergunst et al. 2000). This prey genotype lacks a T6SS-encoding locus and carries a plasmid conferring antibiotic resistance to facilitate selection after competition. The wild-type strains of *A. tumefaciens* suppressed, relative to their corresponding Δ*tssL* mutants, the growth of *E. coli*. However, the strains exhibited varying degrees of efficacy. At one extreme, coculture with wild-type strains C58, 1D1108, and A6 led to an approximately 100× lower relative growth of *E. coli*. In contrast, competition with strains 12D1 and 1D1460 resulted in only a 2 to approximately 10× decrease in relative growth of *E. coli*. Results are consistent with previous findings that *A. tumefaciens*, in a T6SS-dependent manner, can negatively affect growth of *E. coli* in culture.

**Strains vary in the composition of genes downstream of *vgrG*.**

In the downstream portions of the *hcp* loci of all 11 genome sequences, the *vgrG* homologs and associated genes are polymorphic in sequence and composition (Fig. 4A). In strains 1D132, 1D1609, and 1D1460, there are two, three, and one accessory *vgrG* loci or locus, respectively (Fig. 4A). The three strains belong to genomospecies G8, G7, and G4. The amino-terminal portions of the 18 VgrG proteins have a high percentage of identical residues and are homologous to gp27 and gp5 of T4 bacteriophage (Pukatzki et al. 2007; Sheahan et al. 2004). The carboxy-terminal regions are polymorphic in sequence and length, consistent with findings that these regions confer specificity to cognate effector proteins (Bondage et al. 2016). Downstream of nine homologs of *vgrG1* and nine homologs of *vgrG2* are genes encoding either a DUF4123 domain or DUF2169 domain, respectively. These domains are present in the adaptor/chaperone proteins that load effectors to VgrG proteins (Bondage et al. 2016). Members of genomospecies 1 are unusual in that the adaptor/chaperone-encoding gene does not immediately follow *vgrG* (Fig. 4A). We also identified PAAR-encoding sequences in all nine of the *vgrG1*-associated loci. In eight *vgrG2*-associated loci, we predicted PAAR-like DUF4150 domains to be part of a larger protein. In the *vgrG2* locus 2 of 1D1609, the DUF4150 domain is unique, as it is

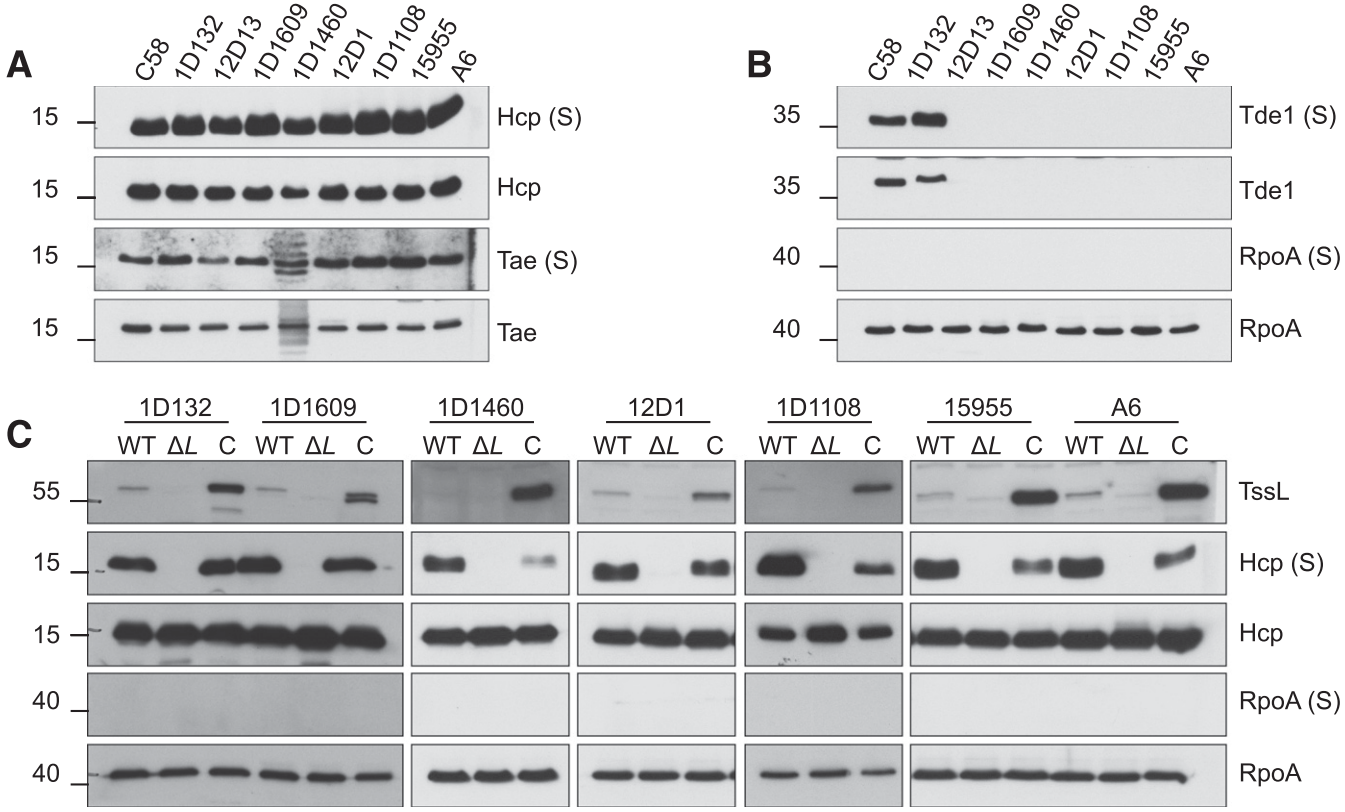


**Fig. 2.** The type VI secretion system is functional in all tested strains of *Agrobacterium tumefaciens*. **A,** Western blots of Hcp and Tae and **B,** of Tde1 and RpoA in soluble media (S) or cell fractions of culture-grown *A. tumefaciens* strains. RpoA is a control for loading and cell lysis. **C,** Western blot of TssL, Hcp, and RpoA in the soluble media (S) or cell fractions of culture-grown *A. tumefaciens* strains. Proteins from strains of wild type with an empty vector (WT), the Δ*tssL* mutant with an empty vector (Δ*L*), or the Δ*tssL* mutant complemented with its allele of *tssL* (lane labeled C) were probed. In all panels, numbers listed along the left indicate the approximate masses in kilodaltons.

predicted to be encoded as a single protein with no other domain. Given the depth of sequencing and that the genome sequences were closed, this gene structure is not likely an artifact of a sequencing mistake.

We next predicted EI pairs and clustered them into 11 families based on sequence similarity (Fig. 4B and C). There are homologs of Tde1 and Tde2, two DNase effectors of strain C58, in 1D132 (Ma et al. 2014). Strain 12D13 has a homolog of Tde2. The prediction of homologs of Tde1 was consistent with findings from western analysis (Fig. 2B). These three strains all belong to genomospecies G8. In strain 1D1609, the four novel E7-E10 genes immediately 3′ to an adaptor gene encode a DUF4150 domain and their C-terminal portions are likely effectors (Fig. 4A). In the *vgrG* locus 2 of strain 1D1609, the E8 gene downstream of the DUF4150-encoding gene is predicted to belong to the Rhs (recombination hot spot) family, which have been associated to T6SS loci in other taxa of bacteria (Hachani et al. 2014; Jones et al. 2014; Koskiniemi et al. 2013; Steele et al. 2017). The putative effector genes, E2 of 1D132, and homologs of E5 in strains 15955 and A6 (as well as Ach5 and H13-3) of genomospecies G1 were relatively easily predicted, because there are only two genes downstream and encoded on the same strand as the adaptor-encoding gene. Strain 12D1 has gene E4, which is predicted to encode a nuclease domain that is present in members of the HNHc superfamily. Members of this family have been associated to effectors of T6SS (Alteri et al. 2013; Zhang et al. 2012). For loci in 1D1460 and 1D1108, there was less information to confidently predict effector genes. Therefore, E3 and E11, respectively, were tentatively assigned as putative effector genes. The genes 3′ to putative effector genes are likely immunity genes. The *vgrG* locus 2 of strain 1D1460 of genomospecies G4 may encode two immunity genes. The translated sequences of the two have 36% amino acid identity. Strain 1D1108 of genomospecies G1 has three genes immediately 3′ to the putative effector gene. The translated sequence of the first (I11_1) has 78 and 82% amino acid identity in comparisons to the translated sequences of the second (I11_2) and third (I11_3) genes, respectively.

The 11 strains can be placed into eight different groups on the basis of their collections of putative EI pairs. Only genomospecies G1 had strains with identical sets of EI gene pairs (Fig. 4A and 4B). Paralleling the variation in EI gene pairs is the variation in *vgrG* as well as adaptor/chaperone and PAAR gene homologs. We considered the composition and relative order of *vgrG*, adaptor/chaperone-, and PAAR- or DUF4150-encoding genes to tentatively classify the *vgrG* loci into at least four variants (Figs. 4A). Most belong to variants 1 and 2, exemplified by the *vgrG1* and *vgrG2* loci of C58. Another variant is defined by the second locus of 1D1609, which has the DUF4150 domain encoded separately from its putative effector gene. Last, is the locus variant present in four of five members of genomospecies G1. Here, the PAAR-encoding gene is upstream of the predicted chaperone-encoding genes. Other minor differences, in 12D1, for example, are a consequence of gain or loss of genes that were disregarded as a criterion in classifying *vgrG* loci. These polymorphic genes are not known to be associated with T6SS function. The duplication of immunity genes was also not considered as a criterion. As such, the locus of strain 1D1108 was classified as variant 2.

### EI pairs are variable in their effects on in-planta competition between strains of *A. tumefaciens*.

Differences in spectra of EI pairs are hypothesized to be predictors of incompatibility between strains. We first tested whether differences in EI pairs between the different species-level groups of *A. tumefaciens* are predictive of outcomes. We compared the level of growth of a prey genotype in competition with a wild-type predator genotype, relative to competition with the corresponding Δ*tssL* mutant of the predator. For genomospecies G8, strain C58 was used as a representative strain and competed 10:1 (predator/prey) against all strains of other genomospecies. The relative growth for each of the six strains was significantly reduced by approximately 0.5 $\log_{10}$ units. For genomospecies G7 (1D1609), G4 (1D1460), and G1 (1D1108), single strains were paired and competed in leaves of *Nicotiana benthamiana* against strains of other genomospecies. In all cases, the growth of the prey genotypes was significantly reduced, varying from a decrease of approximately 0.5 to 1.0 $\log_{10}$ units, relative to growth when paired with the Δ*tssL* mutant of the predator strain (Fig. 5A).

We next tested whether differences in EI pairs are predictive of outcomes of competition between pairs belonging to the same species-level groups of *A. tumefaciens.* The spectra of EI pairs of strains in genomospecies G8 are exact subsets of one another and the predicted EI pairs of strain 12D13 are a subset of those of strain C58, which are a subset of those of strain 1D132 (Fig. 4B and C). Results were largely consistent with expectations (Fig. 5B). When used as prey against strain C58,

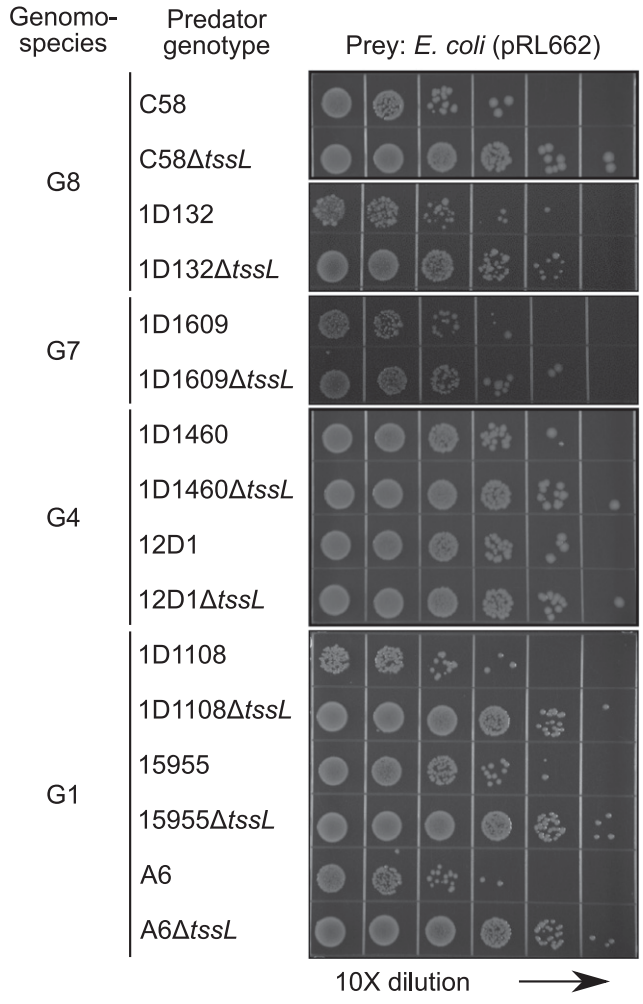


**Fig. 3.** Strains of *Agrobacterium tumefaciens* exhibit type VI secretion system–dependent antibacterial activity against culture-grown *Escherichia coli*. Wild-type strains and Δ*tssL* mutants were mixed 30:1 with *E. coli* and were incubated on lysogeny broth agar for 16 h. Serially diluted cells were grown overnight prior to photographing. Each competition was done at least four times and in three independent experiments. In all cases, C58 and its Δ*tssL* mutant were included as positive and negative controls, respectively. Images were cropped and assembled into a composite figure.

strain 12D13 had a lower amount of relative growth. Strain 12D13 was not tested as a predator because we were not able to make and test its corresponding Δ*tssL* mutant. In a competition of C58 (predator) versus 1D132 (prey), strain 1D132 achieved a higher level of growth relative to competition with the Δ*tssL* mutant of C58. In a competition of 1D132 (predator) versus C58 (prey), the C58 prey achieved lower levels of relative growth. However, results of the three competitions within genomospecies G8 were modest, as none of the results were statistically significant.

In genomospecies G4, it is predicted that the EI pairs of strain 1D1460 differ from the EI pairs of strain 12D1 (Fig. 4B and C). While strain 1D1460 significantly compromised the fitness of 12D1 by 0.5 $\log_{10}$ units (1D1460/12D1), the relative growth of 1D1460 was not affected by 12D1 (12D1/1D1460) (Fig. 5B). The inability of 12D1 to compete could be generalized, as growth of strains that represent each of the three genomospecies was not affected when they were used as prey in competition with 12D1 (Fig. 5C).

In genomospecies G1, it is predicted that the EI pair of strain 1D1108 is predicted to be different from that of strain 15955, which is predicted to be the same as the EI pair of strain A6 (Fig. 4B and C). However, 1D1108 did not affect the relative growth of A6 or 15955 and the relative growth of 1D1108 was not affected by either A6 or 15955 (Fig. 5B). Consistent with expectations, strains 15955 and A6 coexisted in planta, as there were no significant differences regardless of which strain was used as prey.

To address the possibility that strain 12D1 lacks a functional effector, E4 and EI4 of 12D1 were cloned and expressed directly in *E. coli*. When induced, expression of the E4 gene led to a 40% reduction in the growth relative to *E. coli* carrying an empty vector (Fig. 5D). The relative growth of *E. coli* could be rescued by coexpressing the I4 gene. When *E. coli* strains were not induced for expression, their relative growth showed only slight differences relative to a strain carrying an empty vector (Fig. 5D). We could not test the toxicity of E4 directly in *A. tumefaciens*, because the gene could not be cloned without I4 into pTrc200 and failed to yield viable *E. coli*. This is potentially a consequence of leaky expression and toxicity of E4 when expressed from pTrc200, a broad host range vector used for direct and inducible expression in *Agrobacterium* spp. (Schmidt-Eisenlohr et al. 1999). Thus, the data suggested that EI4 of 12D1 functions as an EI pair, which may contribute to the observed antibacterial activity against *E. coli*.

Last, we determined whether any of the three genes downstream of E11 in strain 1D1108 can confer immunity. The direct expression of E11 in the parental 1D1108 strain caused a substantial reduction in growth (Fig. 5E). Coexpression of I11_1, the gene immediately 3′ to E11, led to a partial restoration in growth. I11_2 and I11_3, more distal to E11, showed no measurable protective effect. Thus, we concluded that I11_1 is likely the immunity gene and the two others showed no evidence of conferring immunity in this assay.

## DISCUSSION

We characterized the T6SS from strains that represent four species-level groups of the plant pathogen *A. tumefaciens*. The tested genotypes are genetically diverse and represented lineages within and between species-level groups (Fig. 1). All

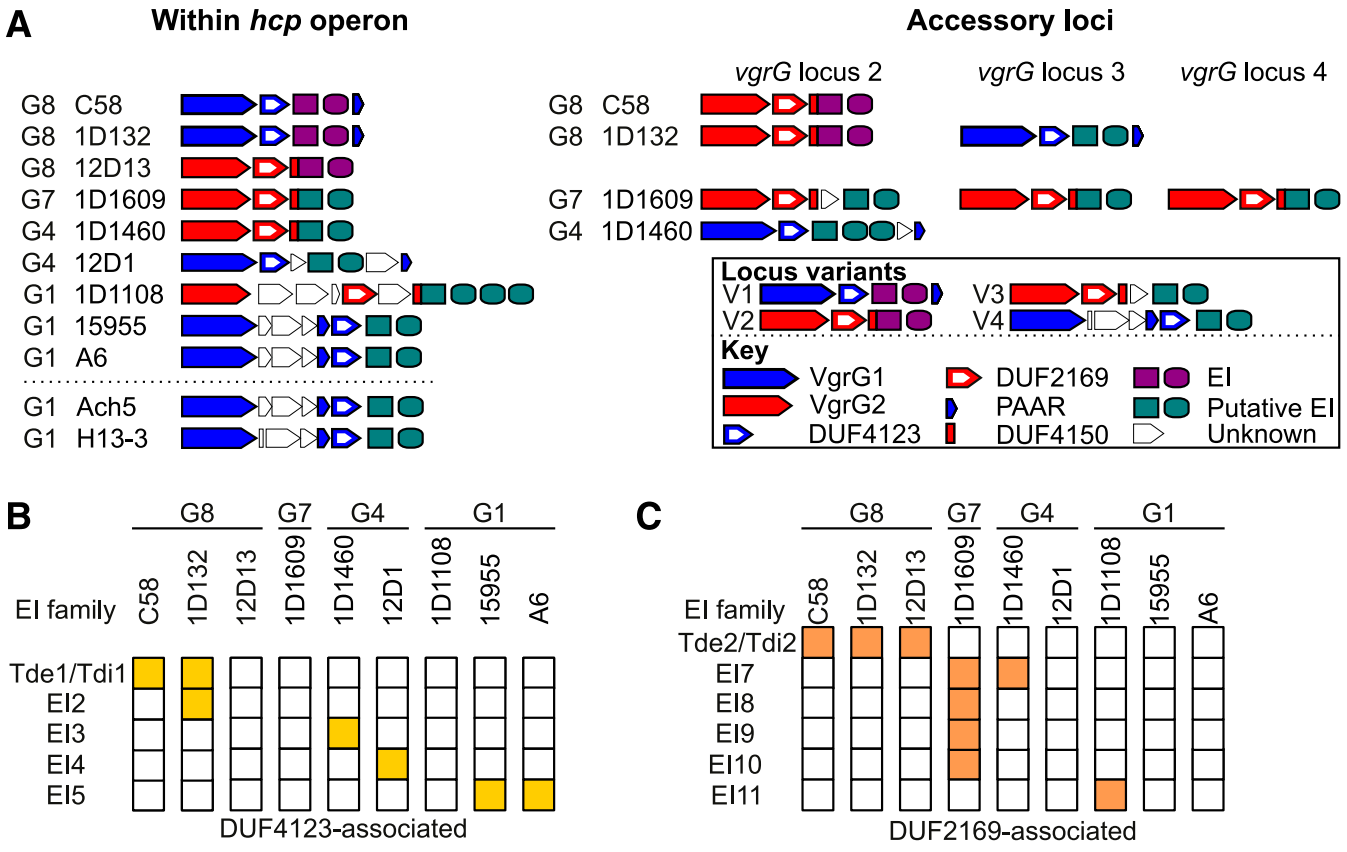


**Fig. 4.** All strains of *Agrobacterium tumefaciens* encode for a type VI secretion system. **A,** Organization of the 3′ portion of *hcp* gene clusters in 11 strains of *A. tumefaciens*; only *vgrG* and downstream genes are shown. Strain names are listed on the left, along with their genomospecies designation. The structures for each of the four locus variants are labeled as *vgrG* locus 2 to 4. A key relating symbols to gene functions is presented in the box. **B** and **C,** Presence (shaded box) or absence (white box) of DUF4123- (B) and DUF2169-associated (C) effector-immunity (EI) pairs in nine strains of *A. tumefaciens*. Genomospecies and strain names are listed above the panels. EI family names are listed along the left of each panel; new putative EI pairs were named EI followed by an arbitrarily assigned number.

strains have *imp* and *hcp* loci and the full suite of genes necessary for regulating and assembling the T6SS. The functionality of the T6SS in secretion and antagonism was confirmed for all tested strains (Figs. 2 and 3). The variation of EI pairs in strains within and between genomospecies is consistent with observations reported for other taxa of T6SS-using bacteria (Fig. 4) (Unterweger et al. 2014).

T6SSs and horizontal gene transfer are intimately linked. T6SS-mediated lysis of bacteria can result in the release of DNA that, when taken up, can integrate into the genomes of surviving cells (Borgeaud et al. 2015). In addition, if fragments of horizontally acquired DNA encode EI gene pairs, there is potential for horizontal gene transfer to diversify the T6SS-associated arsenal (Thomas et al. 2017). None of the species-level groups of *A. tumefaciens* circumscribe strains with identical sets of *hcp* and *vgrG* loci. But in *A. tumefaciens*, several lines of evidence suggest entire *vgrG* modules are likely acquired and selected upon, potentially because of specificity between VgrG, adaptor, effector, and immunity proteins (Bondage et al. 2016). First, in strain C58 and unlike some other species of bacteria, each EI pair is linked to a gene core to the T6SS. Given that a polymutant of C58 lacking all three EI pairs linked to *vgrG* and *hcp* is as compromised in antagonism as the Δ*tssL* mutant, it would suggest that no yet-to-be identified EI pair exists (Fig. 1C) (Ma et al. 2014). Second, when the putative EI pairs uncovered in this study were used as queries, no homologs distal to T6SS-encoding loci were identified. Last, the polymorphisms that were revealed in strains of *A. tumefaciens* were in the composition of genes 3′ to the *tae/tai* EI pair of the *hcp* operon and in the presence or absence of accessory *vgrG* loci (Fig. 4).

Leading models suggest many gram-negative bacteria deploy T6SS to antagonize other bacterial genotypes and species, and the diversification of EI pairs can provide strains a competitive advantage (Ma et al. 2014; Steele et al. 2017; Unterweger et al. 2014; Wexler et al. 2016). Plants are ideal hosts for assessing the role of T6SS during host-associated phases of the life cycle of facultative bacteria. The use of highly inbred lines of plants can help reduce effects of host variation. Even more powerful are the ease to which plants can be manipulated, the low barriers to their use, and the low costs associated with maintenance. We leveraged these strengths to compete almost 30 different pairs of genotypes of *A. tumefaciens*.

Analyses of the large dataset yielded findings that differences in EI pair between strains of *A. tumefaciens* are not the sole factors influencing in-planta competitiveness (Fig. 5). All tested

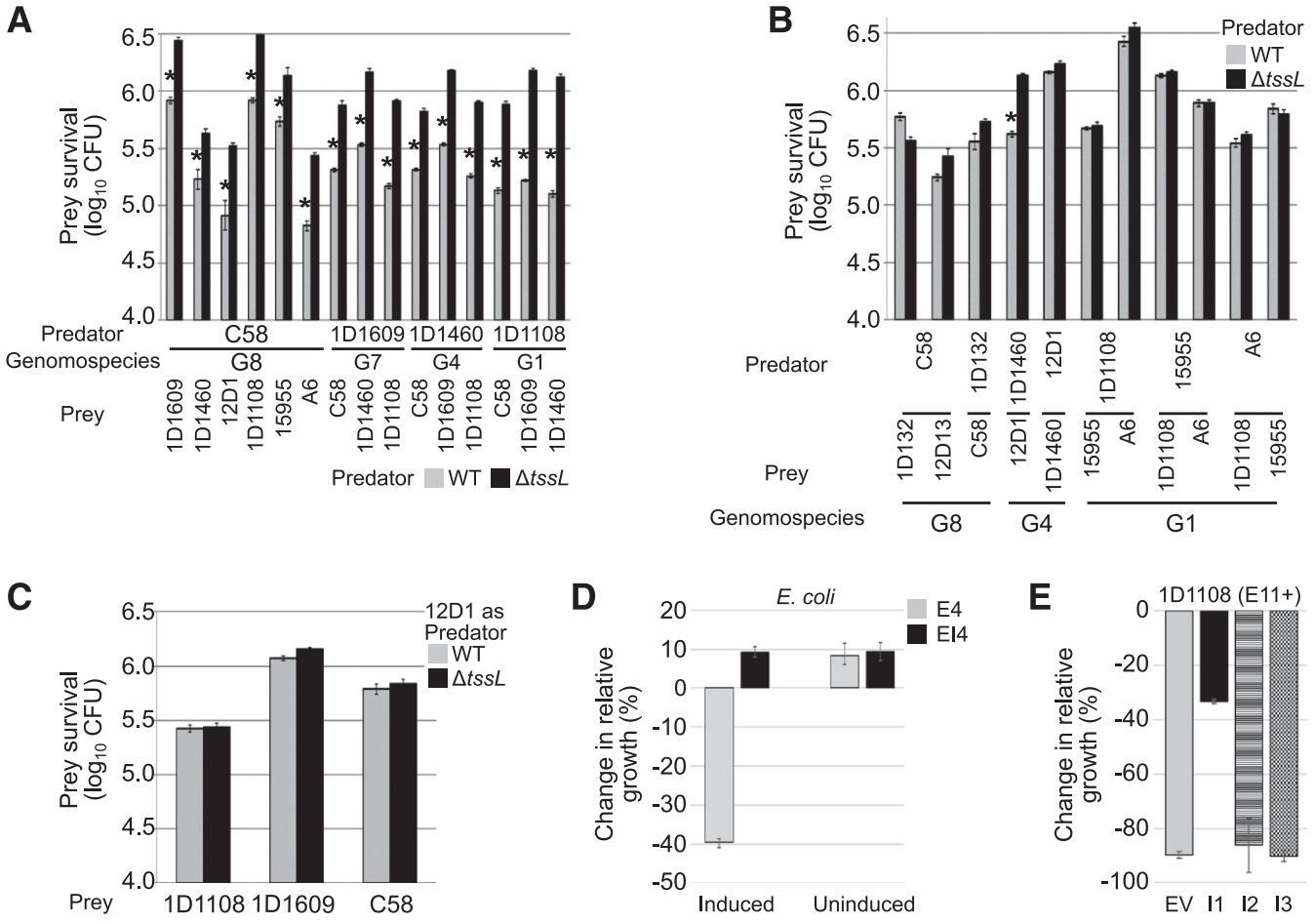


**Fig. 5.** The efficacy of in-planta competition of *Agrobacterium tumefaciens* is variable. **A,** Interbacterial competition between strains of different genomospecies. Wild type or Δ*tssL* mutant strains (predator) representing genomospecies G8, G7, G4, and G1 were competed 10:1 against strains from other genomospecies (prey). After 24 h in planta, recovered prey cells were diluted, plated, and enumerated. **B,** Wild-type strains or Δ*tssL* mutant strains (predator) were competed in planta 10:1 against strains from the same genomospecies. **C,** Strain 12D1 or its Δ*tssL* mutant strain (predator) were competed in planta 10:1 against other strains (prey). After 24 h in planta, recovered prey cells were diluted, plated, and enumerated. Competitions were repeated one additional time with similar results. An asterisk (*) denotes a significant difference ($P$ value ≤ 0.01). **D,** The E4 effector and EI4 pair of strain 12D1 were expressed directly in *Escherichia coli*. Growth changes (optical density at 600 nm [$OD_{600}$]) of cells in media with or without 0.02% L-arabinose were calculated as a percent relative to *E. coli* carrying an empty vector and grown in the same medium. **E,** An empty pRL662 vector (EV) or I11_1-3 of strain 1D1108 were independently paired and expressed directly in *A. tumefaciens* 1D1108 carrying the E11 gene. Growth changes ($OD_{600}$) of cells in media with 1.0 mM isopropyl-β-D-thiogalactoside were calculated as percent relative to the same genotypes carrying an empty pTrc200 vector and grown in the same medium.

*A. tumefaciens* strains exhibited antibacterial activity against *E. coli* but antagonism within species-level groups could not simply be explained on the basis of EI gene pairs. The trend we observed was that partners in intragenomospecies competition tended to exert only minor fitness costs or coexisted regardless of EI incompatibility, while strains mostly exhibited antagonism in intergenomospecies competitions. These findings raise the possibility that genotypic features other than EI gene pairs and associated with phylogenetic lineage can be recognized as cues to trigger efficient T6SS attacks, even prior to toxin-immunity recognition inside prey cells.

However, there are additional factors or features that may have contributed to the low competitiveness observed within the tested strains. The effectors of *A. tumefaciens* may affect only specific genotypes, as species-specific toxicity has been previously reported (Chatzidaki-Livanis et al. 2016; Sana et al. 2016; Schwarz et al. 2010). For strain 12D1, the effector E4 is a putative nuclease that may have low toxicity. When competed against *E. coli*, prey populations showed a minor decrease in growth levels (Fig. 3). Direct expression of the effector in *E. coli* had significant effects, but this may have been a consequence of expression levels (Fig. 5D). However, other strains with weak activity against *E. coli* were nonetheless effective against other strains of *A. tumefaciens*. It is also possible that resistant strains encode orphan immunity genes. If this is the case, the orphan immunity genes are not homologous in sequence, as TBLASTN searches using sequences of immunity genes from 15955, 1D1108, and 12D1 failed to reveal any homologs in the translated genome sequences of strains of *A. tumefaciens* that could coexist with 12D1 in planta. Analyses of whole-genome sequences revealed that, among those in our dataset, strain 12D1 is unique in lacking accessory pAt plasmids. It would be interesting to test whether these accessory plasmids influence the effectiveness of competition.

In addition, modeling and empirical studies show that T6SS-mediated competition is influenced by factors such as density, growth rate, and spatiotemporal partitioning (Borenstein et al. 2015; McNally et al. 2017; Wong et al. 2016). These factors may be more pronounced when bacteria are associated with hosts. Previous findings had demonstrated that *A. tumefaciens* C58 is capable of suppressing, in culture, the growth of *E. coli* (Ma et al. 2014). In contrast, C58 had no measurable competitive effect against a susceptible strain modified to lack all three EI pairs but did when competed in planta. In a different genus of bacteria, it was demonstrated that, in a culture-based assay, strains of *Vibrio cholera* with the same EI gene pairs could coexist, whereas those harboring different EI gene pairs were antagonistic to each other (Unterweger et al. 2014). However, inconsistencies between results derived from cultured-based and host-based assays were recently revealed when *Vibrio fischeri* was examined. Two genotypes of *V. fischeri* incompatible in culture could coexist in squid (Speare et al. 2018). Likewise, a T6SS-using and susceptible genotype could also coexist but did so in different regions of the squid.

Last, variations in bacterial features that are not direct targets of effectors can also affect the efficacy of T6SS competition. Agrobacteria could differ in their ability to colonize and vary in motility, attachment, and ability to form biofilms. Features such as the cell envelope can have different modifications and affect T6SS antagonism. Exopolysaccharide, for example, has been shown to influence susceptibility of *Vibrio cholerae* to T6SS attack (Toska et al. 2018). Any of these traits could impact whether competing genotypes physically interact in a manner that is sufficient to yield quantifiable effects on populations of bacteria.

In summary, findings suggest the effect of T6SS antagonism on population and community dynamics are nuanced. While T6SS-dependent bacterial antagonism was observed in many cases, differences in EI pairs was not always predictive of outcomes. Genetic and ecological factors or the interactions between them are predicted to have significant impacts on T6SS activity. This study establishes a foundation for future studies to inform on the diversity and evolution of EI pairs as more genome sequences become available and more taxa of *Agrobacterium* are characterized. This study also opens future directions to study the ecological role of interbacterial competition and how it influences access to host plants (Bernal et al. 2017).

## MATERIALS AND METHODS

### Plasmids, bacterial strains, and growth conditions.

Wild-type bacterial strains are described in Table 1. Sequences of primers used in this study are listed in Supplementary Table S1.

Strains of *A. tumefaciens* were grown at 28°C in either lysogeny broth (LB) or 523 media. *E. coli* strains K12, DH10B, and *ccdB* survival2 T1 were grown at 37°C in LB (Invitrogen, Carlsbad, CA, U.S.A.). When appropriate, the antibiotics gentamicin, spectinomycin, and kanamycin were added to media at concentrations of 50, 100, and 30 μg/ml, respectively.

### Genome sequencing and analyses.

The procedures for genome sequencing and analysis were based on those described in previous studies (Cho et al. 2018; Huang et al. 2015; Lo et al. 2013). Briefly, one paired-end library and one mate-pair library were prepared for each strain. The shotgun sequencing was performed on the Illumina MiSeq platform with >300× coverage for each library. ALLPATHS-LG was used to de novo assemble reads (Gnerre et al. 2011). PCR and Sanger sequencing were used to fill assembly gaps and to resolve repetitive regions until genome sequences were finished. The gene prediction was based on RNAmmer, tRNAscan-SE, and Prodigal (Hyatt et al. 2010; Lagesen et al. 2007; Lowe and Eddy 1997). The annotation of protein-coding genes was based on the homologous genes in published *Agrobacterium* genome sequences as identified by OrthoMCL (Cho et al. 2018; Huang et al. 2015; Li et al. 2003; Slater et al. 2013). Further manual curation was based on the National Center for Biotechnology Information (NCBI) nonredundant protein database, the NCBI conserved domain database, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and T346Hunter (Benson et al. 2018; Kanehisa and Goto 2000; Marchler-Bauer et al. 2017; Martínez-García et al. 2015). All bioinformatics tools were used with the default settings unless stated otherwise.

Genes associated with the T6SS were identified on the basis of multiple criteria. Sequences of genes from strain C58 were used in queries to identify those core to T6SS (Lin et al. 2013). The annotations and any predicted domains were manually inspected for genes downstream of *vgrG*. Putative EI gene pairs were identified on the basis of location relative to genes core to T6SS and DUF4123- or DUF2169-encoding genes and having a predicted DUF4150 domain.

For comparative genomics, homologous gene clusters were identified using OrthoMCL (Li et al. 2003). Single-copy genes shared by all strains were used for phylogenetic inference. The protein sequences of each homolog were aligned individually using MUSCLE (Edgar 2004) and then concatenated for maximum likelihood analysis using PhyML (Guindon and Gascuel 2003). The proportion of invariable sites and the gamma distribution parameter were estimated from the dataset, the number of substitute rate categories was set to four. The bootstrap supports were estimated based on 1,000 replicates.

**Plasmid construction and bacteria transformation.**

To construct the plasmids for making deletion mutants, fragments of approximately 500 nucleotides in length and flanking target genes were PCR-amplified. In all cases, one flanking fragment was digested with *Xba*I and *Bam*HI, while the other was digested with *Bam*HI and *Xma*I. The two were ligated to pJQ200KS, previously digested with *Xba*I and *Xma*I and were transformed into *E. coli* DH10B (Quandt and Hynes 1993). At least two independent colonies were selected and verified via PCR. Previously described methods were followed to generate in-frame deletion mutants in strains of *A. tumefaciens* (Ma et al. 2009; Wu et al. 2008).

The coding sequences of *tssL* were PCR-amplified. Products were digested with *Xho*I and *Xba*I, were ligated to pRL662 digested with the same restriction enzymes, and were transformed into *E. coli* DH10B (Vergunst et al. 2003). Constructs were verified and those for complementation were electroporated into strains of *A. tumefaciens*.

Effector and immunity genes were cloned into pJN105, pRL662, or pTrc200 (Newman and Fuqua 1999; Schmidt-Eisenlohr et al. 1999; Vergunst et al. 2000). To convert vectors to be compatible with the Gateway system, a pBluescript KS+ plasmid with the RfC.1 cassette was digested with *Sma*I, and the fragment with the cassette was ligated directly into *Sma*I-digested pJN105, *Sma*I-digested pRL662, and *Nco*I-digested and Klenow-treated pTrc200 (Invitrogen). Ligation productions were transformed into *E. coli ccdB* survival2 T1. Restriction digests were used to identify plasmids with the cassette in the correct orientation. The coding sequences of effector or EI pairs were PCR-amplified. Products were recombined to pDONR222 vector via Gateway BP reaction to generate entry clones (Invitrogen). Constructs were verified via PCR. The genes from the entry clones were recombined via Gateway LR reaction into pJN105_RfC.1 and were transformed into *E. coli* DH10B.

**Western blotting.**

For T6SS expression and secretion analysis, *A. tumefaciens* cells were grown overnight, were centrifuged (10,000 × *g*, 10 min), and were resuspended at an optical density at 600 nm ($OD_{600}$) = 0.1 in AB-MES medium (pH 5.5). After shaking for 6 h at 25°C, cells were centrifuged and the supernatant was filtered through a 0.45-µm filter. To precipitate proteins, 30 µl of 1% sodium deoxycholate and 150 µl of trichloroacetic acid was added to 1.0 ml of filtered culture supernatant (Ma et al. 2012). The precipitated proteins were resuspended in 20 µl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer. For the total cell fractions, volumes of SDS-PAGE sample loading buffer were added to normalize fractions to $OD_{600}$ = 5.0. Total volumes of 20 and 5.0 µl of the supernatant and total fractions, respectively, were resolved on SDS-PAGE gels.

Previously described methods for western blots were followed (Lai and Kado 1998). The methods used to generate primary polyclonal antibodies against TssL, Tae, Hcp, Tde1, and RpoA were previously described (Bondage et al. 2016; Lin et al. 2013; Ma et al. 2009; Wu et al. 2008). The secondary antibody used was horseradish peroxidase–conjugated goat antirabbit immunoglobulin G (Chemicon [now EMD Millipore], Billerica, MA, U.S.A.). The Western Lightning System (Perkin Elmer, Boston) was used to detect western blots. In all cases, negative and positive controls were included in blots. Images were cropped and assembled into figures. Experiments were repeated and yielded similar results.

**Culture-based competition assay.**

Previously described methods were followed for the culture-based competition assay (Ma et al. 2014). Briefly, *A. tumefaciens* and *E. coli* K12 cells carrying pRL662, at log phase of growth, were harvested and mixed in LB medium at a 30:1 ratio (*A. tumefaciens/E. coli*). The mixtures were spotted on LB agar plates and were incubated at 25°C for 16 h. Bacteria were scraped, were serially diluted, were plated on LB agar plates containing gentamicin, which selects for *E. coli* cells carrying pRL662, and were grown at 37°C overnight. Experiments were repeated and yielded similar results.

**In planta bacterial competition assay.**

Previously described methods were followed for the in-planta competition assay (Ma et al. 2014). For prey strains carrying pRL662, predator and prey strains were grown overnight, mixed at a 10:1 ratio (predator/prey) in one-half Murashige and Skoog medium (pH 5.7), and were infiltrated with a needleless syringe into the leaves of two different six- to seven-week-old *Nicotiana benthamiana* plants. At 24 h post-infiltration, a number 3 cork borer was used to take three samples from the infiltrated regions. Cores were individually macerated in 1 ml of 0.9% NaCl solution and the samples were serially diluted and plated in triplicate on LB agar plates containing gentamicin. The target strain was quantified by counting CFU. Data were expressed as mean ± standard error. The Tukey honestly significant difference test using the one-way analysis of variance method was used. Experiments were repeated and yielded similar results.

**Expression of T6SS effector and immunity genes in media-grown bacteria.**

Overnight cultures of *E. coli* DH10B cells containing either pJN105::effector gene, pJN105::EI genes, or an empty pJN105 vector were resuspended in 100 µl of LB broth to an $OD_{600}$ = 0.1 with or without 0.02% L-arabinose, and were transferred in triplicate to a 96-well plate. A Tecan Spark 10M plate reader was used to measure the $OD_{600}$ of cultures every hour for 10 h. Cells were grown at 37°C. Growth trends were inspected to ensure bacteria grew as predicted. To calculate growth inhibition, the $OD_{600}$ of cultures at timepoint 8 h were averaged within each treatment and values of *E. coli* cells containing pJN105::effector gene or pJN105::EI genes were calculated as percentage relative to *E. coli* carrying pJN105 growing in the same conditions. Experiments were repeated twice, using $n = 2$, and yielded similar results.

For *A. tumefaciens*, the following modifications were made. *A. tumefaciens* cells contained pTrc200::effector, an empty pTrc200 vector, or pTrc200::effector gene and pRL662::immunity gene. Bacteria were resuspended in a 523 medium with or without 1.0 mM isopropyl-β-D-thiogalactoside. Cells were grown at 28°C.

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