Antieukaryotic Type Six Secretion System Virulence Factors of Bacteria

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Abstract

The type 6 protein secretion system (T6SS) is prevalently utilized by Gram-negative bacteria to compete for resources and space. Upon activation, toxic effectors from this secretion system are translocated into the competitor prokaryote or eukaryote in a contact-dependent manner. While much has been reported on T6SS-mediated prokaryotic competition, very little is understood about the mechanisms of bacterial interactions with eukaryotic hosts. Likewise, many virulent T6SS effectors are known to be antibacterial. In recent years, however, evidence has emerged on numerous T6SS effectors that interact with related immunity proteins in a range of eukaryotic hosts. Insights into how this effector-immunity pairing alters the physiological responses of the recipient organism might provide opportunities relating to the T6SS agricultural and biotherapeutic applications. We, therefore, summarize the impacts of the T6SS effectors with a special focus on bacterial interactions with animals, plants, and fungi. We further briefly discuss pipelines that are currently used to characterize antieukaryotic T6SS effectors.

Keywords: Type VI Secretion System, antieukaryotic effectors, interkingdom competition, virulence
Introduction

Strategies that maximize fitness, survival, and thrive in diverse environments entail numerous common themes in bacteria (e.g., rapid growth and biofilm formation), one of the most important being the type six secretion system (T6SS). This multi-protein secretion system is encoded by large and conserved gene clusters in both pathogenic and non-pathogenic Gram-negative bacteria (GNB)\(^1\), and controls bacterial interaction with other microbes, plants, and animals. The T6SS is highly diverse in animal- and plant-associated bacteria, including the GNB of the phylum Proteobacteria. As a result, phylogenetic studies have sub-classified the system into the main Proteobacterial T6SS\(^I\), Francisella T6SS\(^II\), and Bacteroidetes T6SS\(^III\) cluster loci.\(^2\) The T6SS is analogous to tails of Myoviridae T4 bacteriophages and R-type pyocins, which might have been caused by an endless evolutionary arms-race that governs phage-bacteria interactions, paving way for GNB to repurpose phage gene products integrated into their genomes during infection (Fig 1).

**Figure 1:** Contractile tail bacteriophage and contractile phage tail-like structures. Illustrated in this model are (A) the contractile
Virulence effectors of the T6SS are surprisingly dependent on “themselves” for secretion, lack N-terminal hydrophobic sequence, and sometimes require contact for release.\(^3\) The T6SS and associated effectors shape how bacteria respond to other microorganisms, the host within which they reside, and the environment (Fig 2). This effectively makes the system one of the core regulatory components of interspecies and interkingdom competition in bacteria. However, an in-depth understanding of T6SS-mediated microbe-microbe and host-microbe interactions will come from completing the T6SS structure, which is incomplete due to a lack of high-resolution structural information.\(^4\) As a result, several projects are underway to report on the structures that may provide important steps toward a complete atomic model of T6SS assembly.\(^5\)–\(^7\)

Several reports have established that the T6SS delivers a diverse group of antibacterial and antieukaryotic effectors (Table 1), which promote interspecies and interkingdom communication. Such a communication network appears to be essential for bacteria to respond to fluctuations within dynamic environmental conditions. Beyond this, bacteria, being social organisms, can use T6SS to constantly make physical contact with various eukaryotic hosts over space and time, resulting in either a mutualistic or parasitic lifestyle. A range of conditions can determine whether the former or the latter lifestyle prevails over the other. But with increasing discoveries relating to the participation of secretion system effectors, the molecular exchange of proteins during bacterial-eukaryotic interactions (BEIs) will serve as one of the pivotal determinants of antieukaryotic or symbiotic relationships. The crucial role of molecular mechanisms governing BEIs will likely extend beyond human to plant infecting fungal pathogens in as far as secretion systems are used in defining these interactions.\(^8\)
Table 1: T6SS antieukaryotic and antibacterial effectors.

| T6SS       | Effector          | Species               | Organelle targeted | Antieukaryotic/Antibacterial | Reference |
|------------|-------------------|-----------------------|--------------------|-----------------------------|-----------|
|            | Tae2<sup>BT</sup> | Burkholderia. thailandensis | Y, Peptidoglycan  | Antibacterial               | 9         |
|            | Opi A & OpiB      | Francisella tularensis | N                 | Antieukaryotic              | 10        |
|            | Bte1 & Bte2       | Burkholderia fragilis |                    | Antibacterial               | 11        |
|            | Ssp3, Ssp5, Ssp6 | Serratia marcescens   | Y, Cytoplasmic target, | Antibacterial              | 12        |
|            | Ssp4              | Serratia marcescens   | Y, Periplasmic target | Antibacterial              | 12        |
|            | Rhs1 & Rhs2       | Serratia marcescens   | Y, Cytoplasmic target, Y, DNA | Antibacterial              | 13        |
| H1-T6SS    | Tse1 (Tae1<sup>PA</sup>) | Pseudomonas aeruginosa | Y, Peptidoglycan, | Antibacterial               | 14,15     |
| H1-T6SS    | Tse2              | Pseudomonas aeruginosa | Unknown, Cytoplasmic, Bacteriostatic | Antibacterial              | 14,15     |
| H1-T6SS    | Tse3 (Tge1<sup>PA</sup>) | Pseudomonas aeruginosa | Y, Peptidoglycan  | Antibacterial               | 15        |
|            | Tse4              | Pseudomonas aeruginosa | Y, Membrane       | Antibacterial               | 16        |
|            | VasX              | Vibrio cholerae       | Y, Membrane       | Antibacterial/Antieukaryotic | 17        |
|            | EvpP              | Edwardsiella piscicida | N                 | Antieukaryotic              | 18,19     |
### Edwardsiella ictaluri

| H2-T6SS                   | TpE (Tle4) | Pseudomonas aeruginosa | Y, Membrane & Endoplasmic reticulum | Antibacterial/Antieukaryotic | 20 |
|----------------------------|------------|------------------------|--------------------------------------|-----------------------------|----|
|                            | Vpr00650   | Vibrio proteolyticus   | Y, DNA                               | Antibacterial               | 21 |
|                            | Vpr00050   | Vibrio proteolyticus   | Unknown                              | Antibacterial               | 21 |

| H2-T6SS                   | PldA (Tle5a) | Pseudomonas aeruginosa | Y, Membrane                          | Antibacterial/Antieukaryotic | 22 |
|----------------------------|--------------|------------------------|--------------------------------------|-----------------------------|----|
|                            | Vpr00320     | Vibrio proteolyticus   | Y, Membrane                          | Antibacterial               | 21 |
|                            | Vpr01700     | Vibrio proteolyticus   | Y, Membrane                          | Antibacterial               | 21 |

| H3-T6SS                   | PldB (Tle5b) | Pseudomonas aeruginosa | Y, Membrane                          | Antibacterial/Antieukaryotic | 22 |
|----------------------------|--------------|------------------------|--------------------------------------|-----------------------------|----|
|                            | Vpr02450     | Vibrio proteolyticus   | Y, DNA                               | Antibacterial               | 21 |
|                            | Vpr01580     | Vibrio proteolyticus   | Unknown                              | Antieukaryotic              | 21 |
|                            | Vpr00400     | Vibrio proteolyticus   | Unknown                              | Antieukaryotic              | 21 |
| Tpe1                       | Acinetobacter baylyi ADPI | Y, Periplasm              |                                      | Antibacterial               | 23 |

| T6SS1                     | Vpr01570     | Vibrio proteolyticus   | Y, Actin                             | Antieukaryotic              | 21 |
| Tse1 & Tse2               | Acinetobacter baylyi ADPI | Unknown                  |                                      | Antibacterial               | 23 |
| Tae1                      | Acinetobacter baylyi ADPI | Y, Peptidoglycan         |                                      | Antibacterial               | 23 |
| Tle1<sup>ST</sup>         | Burkholderia thailandensis | Y, Membrane              |                                      | Antibacterial               | 15 |
| Protein | Organism                        | Localization      | Function            | Titer |
|---------|---------------------------------|-------------------|---------------------|-------|
| Tae3<sup>RP</sup> | *Ralstonia pickettii* | Y, Peptidoglycan | Antibacterial       | 24    |
| Tae4    | *Salmonella typhimurium*        | Y, Peptidoglycan  | Antibacterial       | 25    |
| Tae4    | *Enterobacter cloaceae*         | Y, Peptidoglycan  | Antibacterial       | 25    |
| Hcp-ET2 | *Enterobacteriaceae*            | Y, Membrane       | Antibacterial       | 26    |
| Hcp-ET1,3,4 | *Enterobacteriaceae*     | Y, DNA            | Antibacterial       | 26    |
| Tle1<sup>EAEC</sup> | *entero-aggregative Escherichia coli* | Y, Membrane | Antibacterial       | 27    |
| Tge2<sup>pp</sup> | *Pseudomonas protogens* | Y, Peptidoglycan  | Antibacterial       | 28    |
| VgrG3   | *Vibrio cholerae*               | Y, Peptidoglycan  | Antibacterial       | 28    |
| Tle2<sup>VC</sup> | *Vibrio cholerae* | Y, Membrane       | Antibacterial/Antieukaryotic | 29    |
| TseH    | *Vibrio cholerae*               |                  | Antibacterial       | 30    |
| Tle5b<sup>KP</sup> | *Klebsiella pneumoniae*  | Y, Membrane       | Antibacterial       | 31    |
| Tle4-Tli4 | *Pseudomonas aeruginosa*       | Y, Membrane       | Antibacterial       | 32    |
| RhsP1   | *Pseudomonas aeruginosa*        | Unknown           | Antibacterial/Antieukaryotic | 33    |
| PldB    | *Pseudomonas aeruginosa*        | Y, Membrane       | Antibacterial/Antieukaryotic | 22    |
| Azu     | *Pseudomonas aeruginosa*        | N                 |                      | 31    |
| Tle5<sup>KP</sup> | *Klebsiella pneumoniae*  | Y, Membrane       | Antieukaryotic      | 31    |
| RhsA & RhsB | *Dickeya dadantii* | Y, DNA            | Antibacterial       | 34    |
|   | Protein   |     | Target Organism | Function               | Log_2Ratio |
|---|-----------|-----|-----------------|------------------------|------------|
| Tde1 | Agrobacterium tumefaciens C58 | Y, DNA | Antibacterial | 35 |
| Pld1 | Klebsiella pneumoniae | Y, membrane | Antieukaryotic | 31 |
| Tse6 | Pseudomonas aeruginosa | N | Antibacterial | 36 |
| TseC | Aeromonas hydrophila | Y, Membrane | Antibacterial | 36 |
| VgrG1 | Acinetobacter hydrophila | Y, Actin | Antieukaryotic | 37 |
| Slp | Serratia marcescens | Y, Membrane | Antibacterial | 1 |
| TecA | Burkholderia cenocepacia | Y, Actin cytoskeleton | Antieukaryotic | 38 |
| TseL | Vibrio cholerae | Y, Membrane | Antibacterial/Antieukaryotic | 39 |
| KatN | Enterohemorrhagic E. coli | N | Antieukaryotic | 40 |
| VgrG2b | Vibrio cholerae | Y, Membrane, microtubule nucleating complex | Antibacterial/Antieukaryotic | 41 |
| VgrG-5 | Burkholderia thailandensis | Y, Membrane | Antieukaryotic | 42 |
| Tfe2 | Serratia marcescens | Y, Membrane | Antieukaryotic | 43 |
| RhsA | Pseudomonas protegens | Y, DNA | Antibacterial | 43 |
| Tne2 | Pseudomonas protegens | Y, DNA | Antibacterial | 44 |
| Tfe1 | Serratia marcescens | N | Antieukaryotic | 43 |
| TseZ | Burkholderia thailandensis | N | Antieukaryotic | 45 |
| T6SS Type | Gene | Species                        | Feature | Activity                        |
|-----------|------|--------------------------------|---------|---------------------------------|
| T6SS-4    | TseM | *Burkholderia thailandensis*  | N       |                                 |
| T6SS-4    | YezP | *Yersinia pseudotuberculosis*  | N       |                                 |
| H3-T6SS   | TseF | *Pseudomonas aeruginosa*       | N       |                                 |
| T6SS-1    | Tme  | *Vibrio parahaemolyticus*      | Y, Membrane | Antibacterial             |
|           | VgrG4| *Klebsiella pneumoniae*        | N       | Antibacterial/Antieukaryotic     |
|           | MXAN_0050 nuclease | *Myxococcus xanthus* DK1622 | Y, DNA | Antibacterial                  |

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Our understanding of how the transfer of certain compounds such as nutritive metabolites, proteins and nucleic acids, which enable cell-cell communication, facilitate BEIs may be far ahead our understanding of how secretion systems and bacterially secreted effectors contribute to this intricate dialogue. Nonetheless, evidence in support of the role of T6SS in BEIs is still not as forthcoming as for other systems despite the widespread occurrence of T6SS in GNB and the fact that bacteria live in close associations with eukaryotes. Given the anticipated wealth of information on the ecological importance of T6SS, we provide the current state of the knowledge on how this system influences the various collaborative scenarios involving bacteria with animals, plants, and fungi.

### Current understanding of T6SS effectors and functional diversity

The functional spectrum of a secretion system is defined by the proteins it secretes and the outcomes thereafter.\(^{52}\) Different T6SS gene clusters are present in different GNB or in the same bacterium to encode T6SS, that is assembled into a metaphorical "crossbow" consisting of bacterially conserved 13 core structural components.\(^{53}\) These components include TssA-TssM and Proline-Alanine-Alanine-Arginine (PAAR) motif-containing proteins that form the membrane-bound sub-assemblies of one or several complex injection systems. Most of the bacteria encoding the T6SS clusters are regarded as prolific gene exchangers.\(^{54}\) In the case of single-copy clusters, the two major T6SS effector genes, hemolysin co-regulated protein (Hcp), a ring-shaped hexamer that recognizes cognate effectors, and valine glycine repeat G (VgrG), used to puncture neighboring cells, , are commonly found as duplicate or sometimes as more copies in the genome of GNB.\(^{11}\) Clusters that are found within the same organism are not capable of completely substituting one another, although effector redundancy has recently been reported.\(^ {56}\) Therefore, a lack of cluster redundancy may suggest that one organism must employ different T6SS clusters to attack different targets. This way, bacterial interactions mediated by the T6SS can be cooperative, specialized, or competitive. Virulent T6SS effectors can kill both prokaryotic and eukaryotic cells. To this effect, deadly combinations
of specific effectors can be loaded at different positions directly or indirectly via adapters to Hcp, docked to VgrG via the Proline-Alanine-Alanine-Arginine or PAAR motif-containing module, or as functional C-terminals of evolved Hcp, VgrG or PAAR proteins. Additional consequences of functional diversity include opposing views about whether T6SS effectors are that different from toxins secreted by this system. Effector proteins of GNB are defined by their dependency on a secretion system for delivery into eukaryotic targets and exertion of a "subtle" effect on the target cell. In some instances, this occurs within a network of effectors delivered by the same system to suppress or manipulate the target in a certain way beneficial to the pathogen for a time. For example, in the Multiple Effector Translocation VgrG (MERV) model, the transportation of functionally varied combinations of effectors via distinct mechanisms was demonstrated. However, the T6SS effectors targeted at other bacteria may have lethal effects that stimulate reactive oxygen species generation in target cells in a manner similar to when bacteria are exposed to antibiotics. In respect to this mechanism, bacterial T6SS effectors may be associated with "brute force" or lethality, causing irreversible effects and exhibiting high specificity in their biochemical role. Surprisingly, contrary to effector secretion system dependency, the effects of a bacterial toxin can be observed on living cells through exogenous addition. Based on conflicting descriptions and mechanisms, the T6SSs, therefore, appear to secrete toxin-like effector proteins with a variety of cellular activities, including degradative enzymes, transcription factors, and hormones. This suggests the use of both brute force and stealth force by GNB based on arising needs.

Antianimal T6SS effectors

The T6SS is generally perceived to be a dedicated antibacterial weapon (Table 1) (Reviewed in ). However, in addition to antibacterial effectors, discoveries of antieukaryotic, and those that have a dual role called trans-kingdom effectors, have been made. The latter is evidenced by effectors, such as Secreted small protein (Ssp3/Tfe1), which can target both prokaryotic and eukaryotic cells (Table 1). Trans-kingdom effectors possess phospholipase activity and oftentimes target the cell membrane. These
membrane targeting phospholipases form pores that affect cellular integrity (Table1) and include Type VI secretion exported effector L (TseL)/Type six lipase effector 2 (Tle2), Type VI secretion exported effector 5 (Tse5), and Virulence-associated secretion protein (VasX) (Table 1). It is possible that the ability of these effectors to act in trans is due to the existence of ubiquitous target substrates available in both eukaryotic and prokaryotic cell types. Because of minimal interest in T6SS effectors targeted at eukaryotic cells, most of the documented dual effectors are membrane targeting. However, research may uncover effectors belonging to other functional classes. Due to the complexity, versatility, functional diversity, and promiscuity of the T6SS, associated proteins may prove even more varied than presently acknowledged.  

Presumably, not all T6SS effectors are destructive. Some have subtle effects on the host such as manipulating and undermining host defenses. Unlike antibacterial effectors, antieukaryotic effectors do not fall into clear-cut classes. Presently, most T6SS effectors belonging to animal pathogens have been shown to possess the ability to kill host cells and manipulate or avoid their defenses. Numerous studies have presented important evidence that the T6SS does possess antieukaryotic activity against mammalian cells. Interactions between T6SS effectors and eukaryotic cells (BEIs/BAIs) involve manipulation, evasion, and killing, as demonstrated in Fig 2. The eukaryotic targeting T6SS was initially described in Rhizobium leguminosarum, V. cholerae, P. aeruginosa, and Edwardsiella tarda, and identified as a virulence factor involved in interactions with eukaryotes. P. aeruginosa effectors PldA and PldB discharged via the H2 and H3-T6SS respectively are eukaryotic-like phospholipase D (PLD) that contribute to the pathogen’s invasion phenotype. Upon release, PldA degrades phosphatidylethanolamine of other bacteria and eukaryotic membrane, making it a dual targeting/trans-kingdom effector. In other words, this effector serves as a toxic component of P. aeruginosa, ensuring a competitive advantage during the interaction with other bacterial species and to affect host cell integrity. Studies have demonstrated the effects of H2- and H3-T6SS mutations on Caenorhabditis elegans, with both T6SSs contributing to the virulence of P. aeruginosa. Others reported that PldB was the effector of P. aeruginosa H3-T6SS possessing dual targeting characteristics. The VgrG2b effector of P. aeruginosa was shown to interact with γ-tubulin, facilitating bacterial internalization into epithelial cells.
The T6SS-5 of *B. thailandensis* targets eukaryotes and is important for murine pneumonic melioidosis. The C-terminal domain of the T6SS secreted spike protein VgrG1 in *V. cholerae* carries a C-terminal effector domain that cross-links actin, impairing the phagocytic activity of host cells. Taken together, these studies suggest that the T6SS has somehow evolved for competition with diverse eukaryotic hosts (Fig 2).

**Antiplant T6SS effectors**

The virulent role of T6SS protein effectors in phytopathogen-host interactions (BEIs/BPIs) remains a hypothesis supported by findings such as an Hcp protein of *A. tumefaciens* DC58 that facilitates tumorigenesis in potato, and a number of studies showing a decreased virulence phenotype and up-regulation of the T6SS cluster genes during infection of a plant host. For example, recent expression profiling data of *Pectobacterium brasiliense* 1692 shows that upregulation of T6SS at various time points during infection of potato tubers, which suggests a possible function in pathogenesis. It also has been reported that the expression of the T6SS is among several regulatory mechanisms regulated by environmental cues that mimic host conditions. Most T6SSs cannot be used to target Gram-positive bacteria (GPB) because their peptidoglycan is too thick to puncture. However, it is likely that more GPB adapted effectors are yet to be discovered. Based on the size of a plant cell wall, it also begs the question if T6SS effectors have alternative strategies to engage host systems to facilitate their transport to the cell target substrates. In *Xanthomonas oryzae* pv. *oryzicola* GX01, the two T6SS clusters are not required for virulence in rice. Consequently, maybe only antianimal cell T6SS roles exist and the role of the T6SS during plant infection is indirect. Pest invasion and pathogenicity of *Pseudomonas protegens* CHA0, an environmental plant-colonizing bacterium was shown to benefit from T6SS and the two VgrG modules and their respective effectors RhsA and Ghh1. Their conclusion was also that the T6SS dependent alterations in the gut microbiome of the pest insect allowed the bacterium to colonize the insect, and ultimately promote disease. More so, they
expressed curiosity about how such a strategy may be used by the bacterium in its original plant host.

Antifungal T6SS effectors

Evidence in support of bacterial fungal interactions (BFIs) and virulence towards other eukaryotic hosts including plants and animals is only strong for certain secretion systems (e.g., T1SS, T2SS, T3SS, and T4SS, but weak for the T6SS. In some cases, the eukaryotic interaction initiated by the secretion system may have a supportive function for the host fungus to thrive in the environment. This is demonstrated in the endofungal bacteria *Burkholderia rhizoxinica*, where the T3SS maintains the relationship between this bacterium and its host fungus *Rhizopus microspores*. Strains of *B. rhizoxinica* defective in T3SS are unable to reinfect and establish in their host fungus. On the other hand, *R. microspores* strain not colonized by *B. rhizoxinica* due to the T3SS mutation fails to sporulate. However, when *R. microspores* is infected with a wild type of bacterial isolate, the ability of this fungus to sporulate is restored, suggesting the importance of T3SS in the association of the two species. Therefore, secretion systems can confer fitness benefits during BFIs/BEIs, which is something previously thought to be predominantly common during interactions only involving bacterial species. However, a growing body of literature may prompt investigations to search for the T6SS bacterial effectors with antieukaryotic activities.

Perhaps a search for antifungal activity in bacteria in efforts to uncover the role of T6SS could benefit from looking into bacteria with already established antifungal traits. One such species is the Gram-negative rod-shaped bacterium *S. marcescens*. In compost heap microenvironments, *Serratia* strains can quickly spread over the mycelia of zygomycetes but not ascomycetes, possibly as a preference to making associations with fungi of the phylum Zygomycota in the environment. Interestingly, migration of *S. marcescens* is only initiated upon contact of the mycelia with the edges of the bacterial colony, meaning physical contact is key in this association. Of note, knocking out the genes that regulate movement impairs both the spreading of *S. marcescens* and the
killing of the host fungi interacting with this bacterial species. However, the key genes
operating under these regulators (e.g., Serratia phospholipase PhlA and the pore-forming
toxin ShlA) are not involved in the actual killing of the fungi. In addition to that, secreted
S. marcescens chitinases, which are highly produced by this bacterium, were found not
to be involved in the killing of the mycelia. Taken together, these results point to other
mechanisms that could be involved in the killing of fungal cells during the interaction
between S. marcescens and fungi (Fig 2).

Of recent, the T6SS of S. marcescens was strongly implicated in killing the cells of the
ubiquitous yeast Saccharomyces cerevisiae, and human fungal pathogens belonging to
the genus Candida (i.e., C. albicans and C. glabrata). This may partly explain the killing
of fungi of the phylum Zygomycota by S. marcescens as previously discussed. Indeed,
the strongest indications that the T6SS can manipulate BFIs comes from the fact that S.
marcescens directly translocate two T6SS fungicidal effectors, Tfe1 and Tfe2 (Tfe for
T6SS antifungal effector) (Table 1). Initial evidence relating to functioning of these
antifungal effectors suggests that Tfe1 impairs nutrient uptake and amino acid
metabolism and induces autophagy, while Tfe2 impairs membrane potential but without
introducing any pores in this organelle. Further analysis of these T6SS antifungal
effectors will likely provide key insights regarding BFIs in the context of polymicrobial
communities. With the help of tools that are currently being developed, including
bioinformatics approaches as discussed below, we are likely to witness identification of
more T6SS antifungal effectors in future, not just from S. marcescens but other bacteria.
Figure 2: The environmental impact of the type six secretion system (T6SS) entails the regulation of bacterial bacteria interactions (BBIs), bacterial plant interactions (BPIs), bacterial animal interactions (BAIs), and bacterial fungal interactions (BFIs). The T6SS functions also extend beyond these biotic interactions, allowing bacteria to forage key components (e.g. zinc, iron, copper, and manganese) directly from the extracellular environment (BE\textsubscript{I}s).

**Pipelines currently assisting the discovery of antieukaryotic T6SS effectors**

While comparative genomic analysis of the T6SS in sequenced strains has provided information for understanding the distribution and importance of T6SS within species, online bioinformatics databases have been used to annotate homologs of T6SS...
automatically (e.g. SecretEPDB).\textsuperscript{78} Bioinformatics platforms provide a resource for potential effectors using conserved domains/motifs as queries in the databases.\textsuperscript{79,80} Searching for conserved signaling peptides using bioinformatics tools normally ameliorates putative effector discovery. Unfortunately, high diversity and the lack of a conserved N-terminal signature sequence posed a challenge in the identification of T6SS effectors until studies identified N-terminal signal peptide sequences of these effectors, the marker for T6SS effectors (MIX) and Found in type sIX effector (FIX).\textsuperscript{81,82} However, validation of MIX reliability was done against Bastion6, the first machine-learning tool that promises accurate prediction of T6SS effectors.\textsuperscript{83} MIX motif ability in the identification of T6SS effectors or importance for translocation and effector function was very low.\textsuperscript{83} On the other hand, Nguyen et al. used the poorly rated marker and reported 57 novel MIX T6SS effectors and two identified T6SS phospholipases.\textsuperscript{84} Interestingly, the number of the T6SS effectors in \textit{Burkholderia} plant pathogens was higher in comparison to that of plant beneficial bacteria and no MIX candidates were identified in some of the plant-associated beneficial bacteria.\textsuperscript{84} Plant beneficial bacteria may have been expected to have more clusters to confer protection to the plant through bacterial competition. The T6SS effectors in plant pathogens exhibited high likelihood of association with toxicity on their plant host cells.\textsuperscript{84} Preliminary studies of host-pathogen interactions using the Bastion 6 pipeline (Fig 3) identified 13 T6SS candidate effectors of \textit{Pectobacterium parmentieri} RNS08.42.1a\textsuperscript{T} potentially targeted at the plant host but not at other bacteria. \textit{In silico} prediction and functional characterization of identified motifs revealed that the candidate effectors may have potential roles in pathogenic growth and development of disease symptoms. For example, one effector was anticipated to play a role in the degradation of plant cell RIN4 and consequent activation of RPS2 during bacterial infection. Other effectors were candidates for facilitating pathogen evasion of plant immune systems, deactivation of the defense response pathways and mimicking stress signals.

Wang et al. had argued that while fruitful, existing bioinformatics approaches remain restrictive and highly dependent on the existing knowledge of biochemical properties and effector transport mechanisms.\textsuperscript{83} This may be the reason for the lack of confidence but purported existence of antiplant host targeting T6SS effectors. Simplified statistical methods based on sequence similarity, patterns, and linked gene sequence features, also
appear not suited for effector prediction. Based on these conclusions universal machine learning based methods to accurately predict T6SS effector proteins were proposed as a solution. Since 2018, opportunities for further enhancements of bioinformatics tools have been observed. To evaluate existing tools and improve their predictive performance, An et al. built three ensemble models. However, these only outperformed tools for the T3SS and T4SS effector predictors and not for T6SS. Zalguizuri et al. made their contribution by validating a phylogenetic profiling approach for effector identification centered on the co-evolutionary dependence between the secretome and the T6SS. PyPredT6, the python-based prediction tool for T6SS effector protein identification was designed to address the limitations of Bastion6. After evaluating the performance of both Bastion6 and PyPredT6, Wang et al. improved T6SS effector prediction by developing a new tool. They proposed a more powerful and improved ensemble predictor for identifying T6SS effectors with 100% specificity, 99% accuracy, 97.8% Matthew’s correlation coefficient, and 97.1% sensitivity in independent testing. The bioinformatics-based approaches to identify new T6SS effectors are therefore, a convenient and powerful tool for the study of organisms with sequenced genomes and in collaboration with omics technologies (effectomics, genomics, transcriptomics, proteomics, or metabolomics) may facilitate high-throughput identification and functional characterization of candidate effector proteins including those targeting plant hosts.

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**Figure 3:** Schematic depiction of antiplant host T6SS effector discovery. Identification of putative effector candidates using Bastion6, eggNOG mapper and HMMscan on the HMMER package for prediction of type six secreted effectors, functional annotation based on orthology and identification of conserved motifs, respectively.
Conclusions

Scientific ideology that the T6SS is largely antibacterial has been shifted, as we demonstrate in this review, and recently by others. Our challenge, however, is that not only is the T6SS versatile, it is also complicated. Because of its divergent characteristics, there is an accompanying lack of a standard regulatory mechanism or effector repertoire and conduct for the system. This is because each T6SS assumes a certain uniqueness to the next T6SS owing to the structural, target, and mode of action by a diversity of effectors. Therefore, we are yet to make captivating discoveries using analytical methods to study T6SS virulence effector proteins in relation to growth inhibition in eukaryotes. To understand the mechanism and the biological significance of any secretion system, its secreted proteins need to be identified and characterized. This information will enable us to set goals post research findings and strategies for translation of these outputs. Pathogens are studied so that we can either control or exploit them.

There are several possibilities to explore once T6SS effectors targeting eukaryotic hosts such as plants are identified. The T6SS occupies an intersection between eukaryotes, prokaryotes, and the environment, therefore any contribution to the information pool about this system may lead to development of animal, human and crop disease management strategies. In plants, identified effectors that are recognised by resistance or avirulent proteins may be used to identify resistance genes in distant relatives of non-hosts. These can then be expressed in susceptible targets to confer protection. Protein-protein and structural studies can show how the effector and the target interact. Effector-target binding sites can be manipulated to render them non-targets without affecting functions that could be beneficial to the host cell.

A great deal has been achieved in understanding the operating principles of the T6SS and its secreted proteins. However, there is still a lot we do not know about the intricate dialogues it mediates between different bacteria, eukaryotic host, and their respective environments. Moreover, prediction of putative effectors may be improved by coupling machine-learning programs with in vivo expression data, as seen with the prediction of effectors from fungal secretomes. In the foreseeable future, machine learning will be
the mainstream tool for effector discovery. Although computational tools cannot replace experimentation, they can save research funds, time, and resources by providing intel from which informed decisions can be made on a research project. Identified effector candidates can then be easily validated using functional assays.

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**Declaration of interest**

None to declare.

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