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Activity, delivery, and diversity of Type VI secretion effectors

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Abstract

The bacterial type VI secretion system (T6SS) system is a contractile secretion apparatus that delivers proteins to neighboring bacterial or eukaryotic cells. Antibacterial effectors are mostly toxins that inhibit the growth of other species and help to dominate the niche. A broad variety of these toxins cause cell lysis of the prey cell by disrupting the cell envelope. Other effectors are delivered into the cytoplasm where they affect DNA integrity, cell division or exhaust energy resources. The modular nature of T6SS machinery allows different means of recruitment of toxic effectors to secreted inner tube and spike components that act as carriers. Toxic effectors can be translationally fused to the secreted components or interact with them through specialized structural domains. These interactions can also be assisted by dedicated chaperone proteins. Moreover, conserved sequence motifs in effector-associated domains are subject to genetic rearrangements and therefore engage in the diversification of the arsenal of toxic effectors. This review discusses the diversity of T6SS secreted toxins and presents current knowledge about their loading on the T6SS machinery.

KEYWORDS
bacterial competition, bacterial toxins, effectors, protein secretion, T6SS

1 | INTRODUCTION

The bacterial type VI secretion system (T6SS) is a protein secretion apparatus usually composed of a membrane complex that anchors a cytoplasmic tail structurally and functionally similar to contractile injection systems (Taylor et al., 2018; Wang et al., 2019) (Figure 1). In most T6SSs, the membrane complex is composed of at least two inner membrane proteins and an outer membrane lipoprotein assembled in the cell envelope (Rapisarda et al., 2019). The pre-existing membrane complex recruits an assembly platform called the baseplate (Brunet et al., 2015; Nguyen et al., 2017). The baseplate is itself composed of six wedges arranged around a hub provided by the N-terminal domain of the spike protein VgrG (Brunet et al., 2015; Nazarov et al., 2018). The wedges comprise four proteins, including a phage-like receptor-binding protein that is responsible for baseplate anchoring to the membrane complex (Cherrak et al., 2018; Zoued et al., 2013). Once docked to the membrane complex, the baseplate initiates the assembly of a contractile tubular structure by the polymerization of a tail tube surrounded by a contractile

sheath (Basler et al., 2012; Leiman et al., 2009; Zoued et al., 2016). The tail tube-sheath complex assembles in an extended conformation (Basler et al., 2012; Kudryashev et al., 2015; Wang et al., 2017). Its contraction leads to the propulsion of the tail tube across the envelope of the secreting bacterium and penetration into the target cells. The inner tube is topped by the spike that is made of the trimeric VgrG protein and sharpened by a conical PAAR protein. These constitute the needle that is secreted out of the bacterium and delivers the effector proteins (Pukatzki et al., 2007; Shneider et al., 2013; Silverman et al., 2013). The effectors can be translationally fused to needle components, can be recruited to the needle via direct interactions or with help of specific chaperone proteins also known as adaptors (Bondage et al., 2016; Ma et al., 2017a; Pukatzki et al., 2007; Shneider et al., 2013; Unterweger et al., 2017). The effector proteins described to date exhibit a broad variety of different, mostly toxic, activities capable of rapidly eliminating bacterial prey cells or altering the physiology of eukaryotic cells (Cianfanelli et al., 2016b; Durand et al., 2014; Hachani et al., 2016; Russell et al., 2014). To prevent the elimination of kin cells, the antibacterial T6SS toxin activity
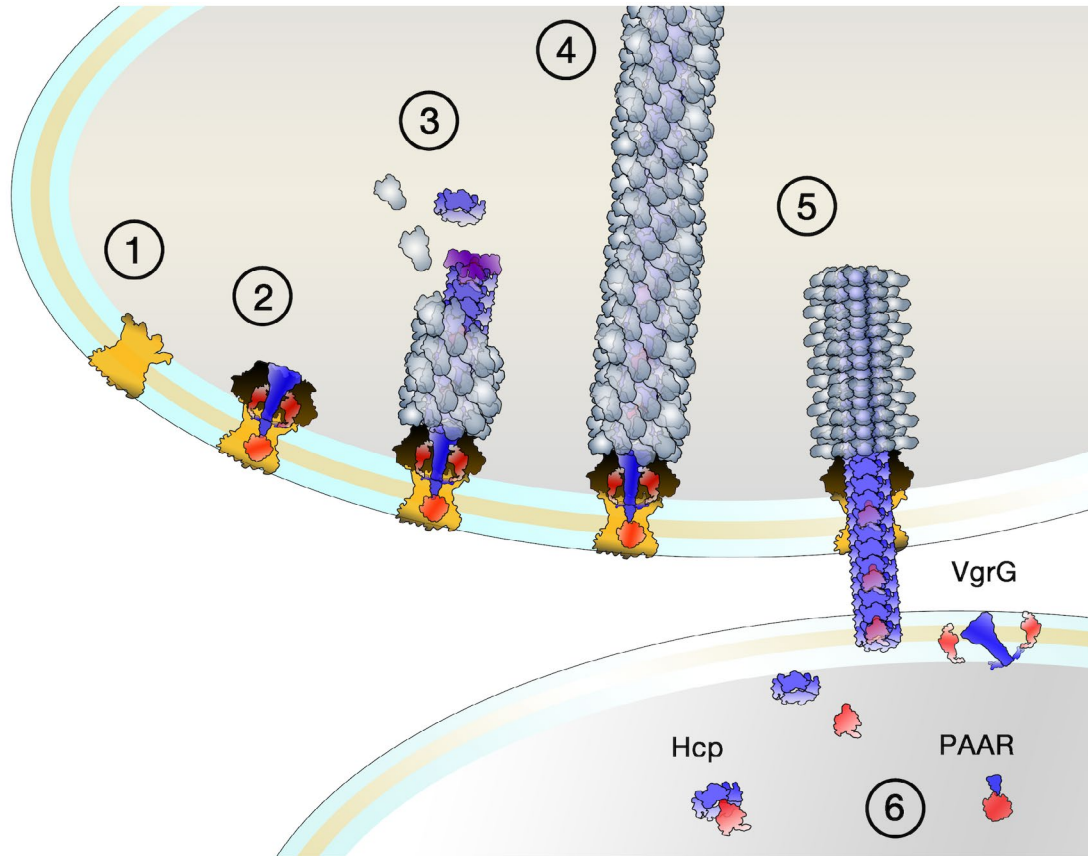
FIGURE 1 Schematic representation of the mechanism of action of type VI secretion machinery. A membrane complex (1) recruits the baseplate (2) which allows the polymerization of the tail tube surrounded by the sheath (3) until it reaches a complete elongated state (4). Upon the contraction of the sheath (5), the inner tube and the spike decorated with effectors are propelled into the target cell (6). The membrane complex is shown in yellow, the baseplate in brown, the sheath in light blue. The secreted components are shown in dark blue— the tail tube consists of a stack of hexameric Hcp rings and spike consists of VgrG and PAAR. The effectors are shown in red

is counteracted by cognate immunity proteins acting as anti-toxins. The acquisition and diversification of the effectors are associated with horizontal gene transfer, gene duplications, and recombination through conserved sequences that locate new toxins and immunities at specific locations in the genome (Koskiniemi et al., 2014; Salomon, 2016; Unterweger et al., 2015). The arsenal of effectors and the stockpile of immunities discussed in this review are key for survival in the ecological niches with intense competition.

2 | ACTIVITY OF T6SS DELIVERED EFFECTORS

In general, the activity of T6SS antibacterial effectors can be subdivided into lytic toxins that exert their activity in the periplasm of the bacterial target and toxins active in their cytoplasm (Figure 2). However, not all proteins delivered by T6SS are toxins, for example, metal-chelating proteins were shown to be expelled through T6SS (Han et al., 2019; Si et al., 2017a; Si et al., 2017b; Wang et al., 2015). Similarly, effectors delivered into eukaryotic cells can alter the prey cells in more sophisticated and subtle manners and therefore deserve a separate discussion (for reviews see Hachani et al., 2016; Monjarás Feria & Valvano, 2020). Some

effectors target molecules that are conserved in both bacterial and eukaryotic cells (e.g., phospholipids or DNA) and are therefore called trans-kingdom effectors. The T6SS delivered repertoire is highly enriched in toxins inducing cell lysis (Smith et al., 2020; Zhang et al., 2012). Achieving rapid lysis of the prey cells can be beneficial in static environments where prey cells can form colonies, as a layer of dead cells would otherwise form a "corpse barrier" and limit access to the remaining prey cells (Borenstein et al., 2015; Smith et al., 2020). Moreover, lysis provides quick access to the released DNA of the prey and can therefore stimulate horizontal gene transfer (Borgeaud et al., 2015; Lin et al., 2019; Ringel et al., 2017). Cytoplasmic acting toxins do not cause rapid cell lysis, nevertheless, most of these toxins are very efficient targeting highly conserved substrates, such as DNA or energy molecules like ATP or NAD+ (Ahmad et al., 2019; Alcoforado Diniz & Coulthurst, 2015; Whitney et al., 2015).

2.1 | Periplasmic acting effectors

2.1.1 | Phospholipases

Type VI lipase effectors (Tle) cause cell lysis of competing bacteria by hydrolyzing phospholipids (Flaugnatti et al., 2016; Jiang et al., 2014;
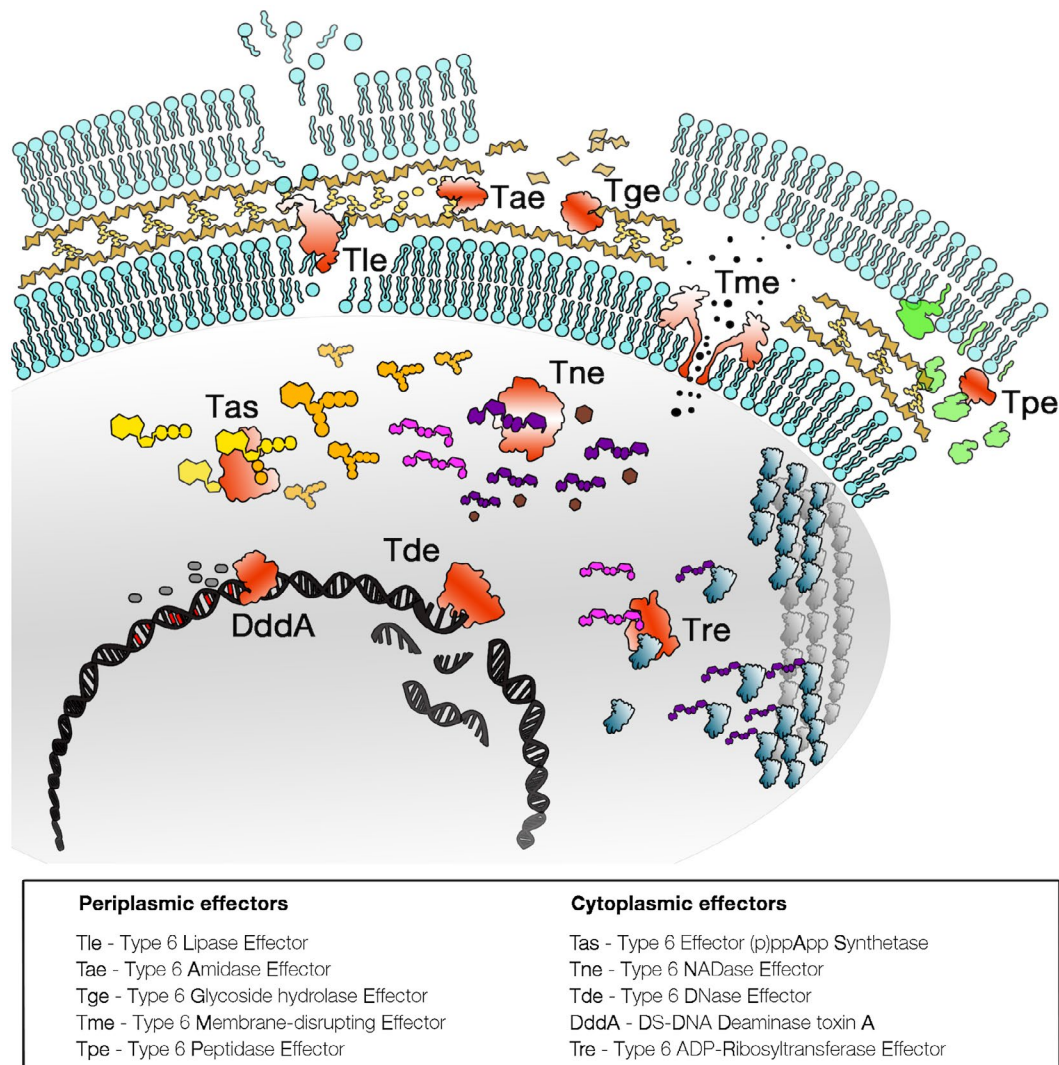
FIGURE 2 Anti-bacterial toxic activities of T6SS effectors. The scheme shows the mode of action of the different families of T6SS toxins. All toxins are shown in red. **Tle** degrade membrane phospholipids (in light blue). **Tae and Tge** effectors degrade the peptidoglycan (in golden) through amidase or glycoside hydrolase activities, respectively. **Tme** form pores in the inner membrane, leading to membrane depolarization (ions in black). One member of the **Tpe** family was proposed to hydrolyze the lipid anchor of outer membrane lipoproteins (in green). **Tas1** phosphorylates ATP and ADP (shown in yellow) leading to the accumulation of (p)ppApp (shown in orange). **Tne** degrade NAD(P) + (shown in pink) to nicotinamide (brown) and ADP-ribose (dark purple), leading to the depletion of NAD(P) + cellular pools. **Tde** catalyze non-specific hydrolysis of DNA (shown in black). **DddA** deaminates cytosine to uracil in the double-stranded DNA. DddA caused mutations are depicted in red, ammonia released as the reaction product in grey. **Tre** ADP-ribosylates protein target (here FtsZ) upon NAD + hydrolysis. FtsZ proteins are shown in blue, NAD + in pink, ADP-ribose in dark purple

Jiang et al., 2016; Ma et al., 2014a; Russell et al., 2013). Tle toxins can be divided into five phylogenetically distinct families named Tle1-Tle5 (Russell et al., 2013). Tle1-4 members hydrolyze bonds between the glycerol and fatty acid tails. They possess phospholipase A_1 activity—cleaving at first (sn1) glycerol position, phospholipase A_2 activity—cleaving at second (sn2) position, or both (Flaugnatti et al., 2016; Hu et al., 2014; Jiang et al., 2016; Russell et al., 2013). These enzymes typically bear a GxSxG motif and a putative S-H-D catalytic triad. All of the currently described Tle effectors comprise domains from AB_hydrolase clan (CL0028), but can encode an additional membrane anchoring domain or domains of unknown function (Berni et al., 2019; Flaugnatti et al., 2016; Hu et al., 2014; Jiang et al.,

2016; Wood et al., 2019b). Tle5 members hydrolyze phospholipids between the phosphate and the head group (at the sn3 position) and therefore possess phospholipase D activity. They are structurally distinct from Tle1-4 and bear dual HxKxxxxD motifs (Jiang et al., 2014; Russell et al., 2013; Spencer & Brown, 2015). Cleavage of phospholipid tails causes shrinking, inflating, and eventual lysis of the target cell (Ringel et al., 2017). The Tle5 activity affects the phospholipid composition and can be toxic due to the accumulation of phosphatidic acid generated by cleavage of the head groups of phospholipids (Lery et al., 2014; Russell et al., 2013). In addition to their antibacterial activities, some Tle can target eukaryotic cells and act as transkingdom effectors. The *Pseudomonas aeruginosa*

PldB/Tle5b effector facilitates intracellular invasion of eukaryotic cells by the activation of the PI3 kinase pathway, while TplE/Tle4 activity leads to endoplasmic reticulum disruption, triggering autophagy (Jiang et al., 2014; Jiang et al., 2016).

2.1.2 | Membrane disrupting/pore-forming toxins

T6SS membrane disrupting effectors (Tme) or pore-forming toxins exert their toxicity from the periplasm of the target cells, by inserting into the inner membrane and causing membrane depolarization. Unlike other effectors, Tme effectors do not possess catalytic activity and induce growth inhibition rather than cell lysis (Fridman et al., 2020; LaCourse et al., 2018; Mariano et al., 2019; Miyata et al., 2013). These effectors share homologies with pore-forming colicins or have predicted transmembrane helices (TMH) in their C-terminal region (Fridman et al., 2020; LaCourse et al., 2018; Miyata et al., 2011). These TMH can bear glycine zipper motifs (LaCourse et al., 2018), a motif commonly found in channel proteins or amyloid proteins and known to promote homo-dimerization (Kim et al., 2005; LaCourse et al., 2018). *P. aeruginosa* Tse4 and *Serratia marcescens* Ssp6 effectors form ion-selective pores, but are not permeable to larger compounds (LaCourse et al., 2018; Mariano et al., 2019). In contrast, the activities of VasX and *Vibrio parahaemolyticus* Tme1 and Tme2 induce permeability to larger compounds such as propidium iodide or ONPG (Fridman et al., 2020; Miyata et al., 2013). However, the precise molecular mechanisms underlying pore formation and/or membrane disruption induced by these different toxins remain to be determined.

2.1.3 | Peptidoglycan hydrolases

A broad spectrum of T6SS effectors causes cell lysis by degrading peptidoglycan. They can be subdivided into those that hydrolyze the glycan backbone (glycoside hydrolases, Tge family) and those that hydrolyze the peptide sidechains (amidases or peptidases, Tae family). Tge effectors hydrolyze the N-acetylmuramic acid (NAM)-N-acetylglucosamine (NAG) glycan strands (Hood et al., 2010; Ma et al., 2018; Russell et al., 2011; Whitney et al., 2013). *Pseudomonas protegens* Tge2 presents a lysozyme-like fold and is neutralized by an immunity protein similar to proteinaceous lysozyme inhibitors (Whitney et al., 2013). In some cases, a lysozyme-like effector is fused to VgrG, such as in *Vibrio cholerae* VgrG3 (Brooks et al., 2013; Dong et al., 2013b). This is reminiscent of the VgrG homolog gp5 from phage T4 which also carries a lysozyme domain to facilitate cell penetration. (Leiman et al., 2009). The Tae (for Type VI amidase effector) family of peptidoglycan hydrolases cleave the peptide stems or the peptidoglycan cross-links. They bear different cleavage bond specificities: Tae1 and Tae4 are D,L-endopeptidases whereas Tae2 and Tae3 are D,D-endopeptidases (Russell et al., 2011; Russell et al., 2012; Srikannathasan et al., 2013). In addition, amidase effectors specific to the NAM-L-Ala[1] bond have been characterized (Ma et al., 2018; Wang et al., 2020). Recently, a novel *Salmonella*

enterica effector Tlde1 (Type 6 L-D-transpeptidase effectors) was characterized. It comprises an L-D transpeptidase-like domain. However, Tlde1 does not crosslink NAM-NAG tetrapeptides, but retains L,D-transpeptidase exchange activity (exchanging D-Ala[4] by a non-canonical D-amino-acid) and possesses an additional L,D-carboxypeptidase activity, cleaving tetrapeptides between mDAP[3] and D-Ala[4]. These modifications in peptide stems impair the biosynthesis of the peptidoglycan (Sibinelli-Sousa et al., 2020).

2.1.4 | Metallopeptidases

Periplasmic-acting Type VI peptidase effectors (Tpe) belong to zinc-dependent metallopeptidases (Peptidase_MA, CL0126) that bear a HExxH zinc-binding motif (Wood et al., 2019a; Zhang et al., 2012). *P. aeruginosa* Tpe1 is not active against peptidoglycan and was rather suggested to cleave the lipoprotein anchors since it induced release of lipoproteins from the membrane (Wood et al., 2019a). A large variety of predicted T6SS peptidase effectors may present diverse activities against periplasmic as well as cytoplasmic targets (Ma et al., 2017b; Zhang et al., 2012).

2.2 | Cytoplasmic acting effectors

2.2.1 | DNases

Most of the T6SS DNase effector (Tde) toxins belong to the HNH superfamily, also known as His-Me finger endonucleases (Alcoforado Diniz & Coulthurst, 2015; Koskiniemi et al., 2013; Pei et al., 2020; Pissaridou et al., 2018; Zhang et al., 2012). A His-Me finger is a short 30-amino acid motif formed by a β-hairpin followed by an α-helix (ββα) that constitute a binding site for a divalent metal ion (Jablonska et al., 2017). The His-Me finger fits its α-helix into the DNA minor groove aligning the β-hairpin with the catalytic histidine against the DNA backbone in a conformation efficient for non-specific DNA hydrolysis. Although additional structural motifs can provide specificity for nucleic acid structure or sequence (Jablonska et al., 2017), no sequence specificity has been demonstrated for any Tde proteins. Target cells exposed to DNase toxins form filaments due to the induction of the SOS response and eventually lose DAPI staining, indicating complete degradation of chromosomal DNA (Jana et al., 2019; Ma et al., 2017a; Pissaridou et al., 2018). Apart from the HNH family, other DNase domains have been identified in Tde effectors, such as those belonging to families PoNe, Ntox, and Tox-Rease (Bernal et al., 2017; Bondage et al., 2016; Jana et al., 2019; Ma et al., 2014b; Zhang et al., 2012). The HNH motif in the Tde effector from the uropathogenic *Escherichia coli* is preceded by an additional Pyocin_S DNase domain (Ma et al., 2017a; Nipic et al., 2013). This effector is active against eukaryotes, where toxicity manifests in typical signs of DNA-damage provoked apoptosis such as cell rounding and membrane blebbing (Nipic et al., 2013).

2.2.2 | Deaminases

Numerous families of deaminase toxins associated with the T6SS have been predicted (Iyer et al., 2011; Zhang et al., 2012). Recently, an example of deaminase of the SCP1.201-like family has been characterized (Mok et al., 2020). *Burkholderia cenocepacia* DddA toxin catalyzes the deamination of cytosine to uracil in double-stranded DNA. The generated uracil is then a substrate for base excision repair and results in C-G to T-A base pair transition (Mok et al., 2020). DddA toxin shows a preference for cytidine bases preceded by thymidine (5′-TC), and due to such modest specificity, it is extremely mutagenic (Mok et al., 2020).

2.2.3 | NAD-glycohydrolases (NADases)

NAD(P) + glycohydrolase toxins (Tne, for Type 6 NADase Effector) degrade NAD(P) + to nicotinamide and ADP-ribose at the rate of ten to one hundred thousand molecules per minute, therefore, quickly depleting the cellular pools of essential NAD(P) + coenzymes primarily used for redox reactions (Tang et al., 2018; Whitney et al., 2015). Drastically reduced NAD(P) + concentrations in the intoxicated cell lead to general growth inhibition with cells maintaining structural integrity (Whitney et al., 2015).

2.2.4 | (p)ppApp synthetases

Tas1 effector secreted by a virulent clinical isolate of *P. aeruginosa* structurally resembles RSH domain (RelA-SpoT homolog) enzymes that synthesize (p)ppGpp, a signaling molecule that responds to nutritional stress (Ahmad et al., 2019; Hauryliuk et al., 2015). Typical RSH enzymes phosphorylate GDP or GTP to yield ppGpp. Tas1, however, is a pyrophosphate kinase for adenosine nucleotides that phosphorylates ATP, ADP or AMP (Ahmad et al., 2019). Due to a high synthesis rate, reaching up to 180,000 phosphorylated ATP molecules per minute, attacked cells are quickly depleted in ATP and ADP and accumulate (p)ppApp (Ahmad et al., 2019). ATP and ADP depletion have a pleiotropic impact on energy production as well as anabolic and catabolic processes. In addition, (p)ppApp resembles (p)ppGpp and blocks purine synthesis by direct interaction with PurF (Ahmad et al., 2019). The accumulation of (p)ppApp could also contribute to toxicity by binding a multitude of other (p)ppGpp targets (Ahmad et al., 2019).

2.2.5 | ADP-ribosyltransferase effectors

Serratia proteamaculans Tre1 is an antibacterial ADP-ribosyltransferase, transferring the ADP-ribose moiety on protein targets upon hydrolysis of NAD + (Ting et al., 2018). Tre1 comprises a characteristic RSE-motif common to ADP-ribosyl transferases. It exclusively ribosylates arginine residues and its main target is FtsZ—a

bacterial tubulin-like protein essential for cell division. Ribosylated FtsZ fails to polymerize, and hence cells are unable to septate, form filaments, and eventually lyse (Ting et al., 2018). Another example of T6SS ADP-ribosyltransferase effector is the *Aeromonas hydrophila* VgrG1 C-terminal domain that targets the eukaryotic cytoskeleton (Suarez et al., 2010). The C-terminal domain of VgrG1 ribosylates actin and impedes its polymerization, leading to cell rounding, activation of caspases, and eventually apoptosis (Suarez et al., 2010).

2.3 | Extracellular effectors

As a metal scavenging strategy T6SS can secrete metallophore effectors directly into the extracellular medium. This secretion strategy is contact-independent and allows metal acquisition. Proteins with binding specificity for zinc, manganese, and copper have been shown to be secreted by T6SS (DeShazer, 2019; Han et al., 2019; Si et al., 2017a; Si et al., 2017b; Wang et al., 2015). *P. aeruginosa* secreted TseF effector does not bind metals directly but binds the iron-loaded extracellular signaling molecule PQS (Pseudomonas Quinolone System) (Lin et al., 2017). Scavenged metals are then imported back to the secreted bacterium by specific TonB-dependent transporters and provide a growth advantage in scarce conditions in the host or in the environment or are used for specific purposes such as oxidative stress resistance (Lin et al., 2017; Si et al., 2017a; Si et al., 2017b).

3 | IMMUNITY PROTEINS

To protect themselves from intoxication T6SS[+] bacteria possess immunity genes adjacent to the cognate toxin genes. As a rule, the immunity proteins protecting from periplasm-active toxins are themselves located in the periplasm, being membrane-anchored lipoproteins, periplasmic, or inner membrane proteins (Flaugnatti et al., 2016; Fridman et al., 2020; Russell et al., 2013; Wood et al., 2019a). Effectors can also be encoded without adjacent immunity genes when they are active against targets not found in the effector-producing bacterium, for instance, targets only found in eukaryotes (Zhang et al., 2012). Importantly, some bacteria can be intrinsically resistant to the anti-bacterial toxins due to the absence or difference of the target or due to specific responses (Hersch et al., 2020; Kamal et al., 2020; Le et al., 2020). Most immunity proteins are small single-domain proteins that typically engage in highly specific interactions with their cognate toxins and occlude their active sites (Ding et al., 2012; Dong et al., 2013a; Robb et al., 2016; Wang et al., 2013; Whitney et al., 2013; Zhang et al., 2012; Zhang et al., 2013a; Zhang et al., 2013b), or lock them in a dysfunctional conformation (Lu et al., 2014). However, some immunities have a dual function, as demonstrated for Tri1, which binds the Tre1 toxin and also removes the ADP-ribosylation from toxin-modified proteins via its ADP-ribosyl hydrolase domain (Ting et al., 2018). As a result, Tri1 immunity was shown to be rather promiscuous, able to neutralize

various ADP-ribosylating toxins (Ting et al., 2018). Interestingly, multiple immunity genes can be organized into poly-immunity loci that are thought to permit "cheating" in ecosystems charged with different bacterial species such as soil or microbiome (Barretto & Fowler, 2020; Kirchberger et al., 2017; Ross et al., 2019; Zhang et al., 2012). The acquisition of new immunity genes is associated with horizontal gene transfer and recombination (Kirchberger et al., 2017; Zhang et al., 2012).

4 | EFFECTOR LOADING AND DELIVERY

T6SS effectors can be translationally fused to secreted T6SS core components—VgrG, PAAR or Hcp. Otherwise, when encoded as separate genes, the effectors locate to VgrG, PAAR or Hcp through specific interactions. These interactions can be assisted by dedicated chaperones or structural motifs (Ma et al., 2017a; Shneider et al., 2013; Silverman et al., 2013; Unterweger et al., 2015; Unterweger et al., 2017) (Figure 3).

4.1 | Core components carrying effectors

The VgrG spike protein forms trimers consisting of a cylinder-shaped base followed by a β-helical prism extension (gp5 needle-like domain) (Spinola-Amilibia et al., 2016; Uchida et al., 2014). The gp5 needle domain can be followed by additional effector domains

or effector-recruitment domains (Flaugnatti et al., 2016; Pukatzki et al., 2007). PAAR domain proteins form cone-shaped structures (Rigard et al., 2016; Shneider et al., 2013). The base of the cone complements the C-terminal β-strands of VgrG; however, the termini of PAAR are surface exposed and therefore are apt for extensions and are often C-terminally fused to various effector domains as well as recruitment domains (Rigard et al., 2016; Shneider et al., 2013). Hcp proteins form hexameric rings that are stacked to build a tube upon sheath formation. The central channel of an Hcp ring has a diameter of roughly 40 Å which can accommodate small effector proteins (Mougous et al., 2006; Silverman et al., 2013). Additionally, Hcp can also provide stability for its effectors and thus may contribute to chaperone functions (Silverman et al., 2013).

Different effector groups may prefer certain types of core component carriers. For example, phospholipase effectors are usually transported by VgrG (Russell et al., 2013). Likely, due to their relatively large size (60–100 kDa), they would not fit inside the lumen of the Hcp tube (Flaugnatti et al., 2016; Flaugnatti et al., 2020; Liang et al., 2015; Ma et al., 2020; Unterweger et al., 2015; Wettstadt et al., 2019). Nevertheless, some Tle effectors fused to PAAR or Hcp were identified and await characterization (Flaugnatti et al., 2020; Ma et al., 2017a). In contrast, cytoplasmic acting toxins are almost exclusively fused to PAAR, Hcp or specialized recruitment domains (Ahmad et al., 2019; Bernal et al., 2017; Jana et al., 2019; Koskiniemi et al., 2013; Ma et al., 2017a; Mok et al., 2020; Pissaridou et al., 2018; Tang et al., 2018; Ting et al., 2018). Structural motifs encoded
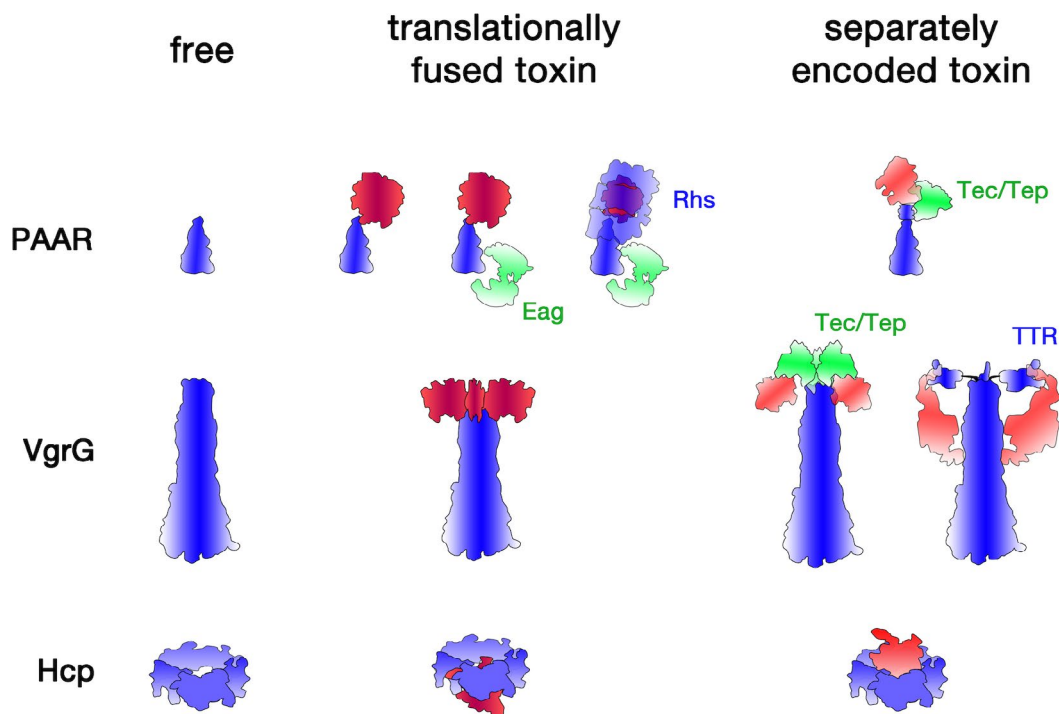

FIGURE 3 Currently described modes of effector loading on VgrG, Hcp or PAAR. Effectors (shown in red) can be fused to Hcp, VgrG or PAAR components or be encoded separately and interact with these components directly or with the help of chaperones/adaptors. Chaperones can be either encoded separately (shown in green) or constitute recruitment motifs that are integral parts of VgrG or PAAR. Chaperones (Eag, Tep/Tec), Rhs domain, as well as a TTR domain are indicated

in these domains might facilitate the translocation of these toxins to the cytoplasm of the prey.

4.2 | Adaptors/chaperones

T6SS chaperones (also called adaptors) are accessory proteins that share several common features—they are encoded adjacent to the toxin and are specific and indispensable for the secretion of their cognate effector, although not secreted themselves (Bondage et al., 2016; Cianfanelli et al., 2016a; Unterweger et al., 2015; Whitney et al., 2015). While these proteins may facilitate proper folding of the effector, their main function is to load their cognate toxin on the T6SS elements.

Eag (for Effector associated gene) family chaperones comprise a DcrB (previously DUF1795) domain. They are required for complex formation between VgrG and their specific cognate N-terminal PAAR-domain containing effectors (Cianfanelli et al., 2016a; Quentin et al., 2018; Whitney et al., 2015). Eag family chaperones are often encoded adjacent to evolved PAAR-toxins that bear transmembrane domains and provide stability by direct interactions that cover the TM domains (Quentin et al., 2018). Specific Eag chaperones encoded in tandem with different toxins can recruit them to the same VgrG spike and therefore facilitate the diversification of effector load (Cianfanelli et al., 2016a).

Agrobacterium tumefaciens Atu3641 is required for the secretion of the PAAR-fused Tde2 DNase. It is so far the only example of DUF2169-containing chaperone family that seems to be associated with the secretion of PAAR-fused effectors (Bondage et al., 2016).

Tec/Tap family adaptors specifically interact with toxic effectors and with VgrG extensions or PAAR extensions (Bondage et al., 2016; Burkinshaw et al., 2018; Liang et al., 2015; Unterweger et al., 2015). The *P. aeruginosa* TecT chaperone additionally requires a co-chaperone that is displaced upon binding to PAAR protein extension (Burkinshaw et al., 2018). Tec/Tap proteins share a structurally uncharacterized DUF4123 domain (Liang et al., 2015; Unterweger et al., 2015). These chaperones are encoded upstream of their cognate effectors and have C-terminal ends specific to the N-terminal domains of their cognate effectors (Liang et al., 2015; Unterweger et al., 2015). The central regions of these chaperones are highly conserved and were proposed to be involved in recombination generating diverse chimeras (Unterweger et al., 2015).

Finally, *P. aeruginosa* DUF2875-containing Tla3 protein interacts with the Tle3 effector and with the C-terminal extension of VgrG2b and was proposed to belong to a novel T6SS chaperone family (Berni et al., 2019).

4.3 | Recruitment domains and motifs

While chaperones are usually stand-alone proteins, some domains decorating the VgrG-PAAR spike perform a similar function, likely related to effector delivery. They range from smaller domains like MIX (Marker for type sIX effectors) or FIX (Found in type sIX effectors) sequence motifs, TTR (Transthyretin-like domains) or DUF2345 domains to larger structures like Rhs (Rearrangement hot spot) core domains.

MIX and FIX are N-terminal sequences preceding the toxic domains in some T6SS effectors, but can also be found embedded into VgrG or PAAR proteins (Jana et al., 2019; Salomon et al., 2014). MIX are predicted to be primarily β stranded structures that consist of a highly hydrophobic central motif and less conserved termini (Salomon et al., 2014). MIX domains are of modular nature, can be duplicated or associated with transposable elements, which suggests they serve for effector pool diversification (Salomon, 2016; Salomon et al., 2014). TTR domains exist as C-terminal VgrG or PAAR extensions and were shown to bind effectors (Flaugnatti et al., 2016; Flaugnatti et al., 2020; Shneider et al., 2013; Wettstadt et al., 2019; Wood et al., 2019b). Bacterial TTR-like domains are globular structures composed of two small β-sheets structurally resembling the eukaryotic TTR β-sandwich structure (Flaugnatti et al., 2020). In enteroaggregative *E. coli* VgrG^{EAEC}, the TTR is fused to the VgrG gp5 needle-like domain through highly flexible loops (Flaugnatti et al., 2020). TTR interacts with the flexible N-terminal extension of the Tle1 effector which inserts its β-sheets to form a β-barrel with TTR (Flaugnatti et al., 2020). In addition to the recruitment function, TTRs can also provide stability to the effectors as well as participate in effector neutralization (Flaugnatti et al., 2020; Wettstadt et al., 2019). DUF2345 domain forms an extension of VgrG by continuing a β-prism structure of the C-terminal gp5 domain of the VgrG spike (Flaugnatti et al., 2020). DUF2345, as well as long α-helices aligned against the length of the needle, make numerous contacts with loaded effector. Together with the TTR domain, they can be considered an internal adaptor domain for the direct recruitment of the effectors (Flaugnatti et al., 2020). DUF2345 domains can perform additional functions. In *Acinetobacter baumannii*, the DUF2345 of VgrG is essential for T6SS assembly, while the VgrG4 DUF2345 domain of *Klebsiella pneumoniae* is itself an antibacterial and antifungal toxic effector inducing response to reactive oxygen species (Lopez et al., 2020; Storey et al., 2020). Rhs are large proteins that were first identified as recombination-prone loci in chromosomes (Lin et al., 1984). Certain classes of Rhs proteins are secreted through T6SS (Alcoforado Diniz & Coulthurst, 2015; Koskiniemi et al., 2013; Pei et al., 2020). They are encoded downstream of VgrG or T6SS chaperones (Eag or Tec/Tap) and comprise N-terminal PAAR or other VgrG interacting (VIRN) domains (Cianfanelli et al., 2016a; Pei et al., 2020). The Rhs core region comprised of spiralling β-sheets forms a shell-like structure that likely encloses the C-terminal toxin domain (Alcoforado Diniz & Coulthurst, 2015; Jackson et al., 2009; Jackson et al., 2019; Zhang et al., 2012). It has been recently demonstrated that a T6SS secreted Rhs protein undergoes two autocleavages—one at the N-terminus and one at the C-terminus (Pei et al., 2020). These conserved autocleavages were suggested to be a part of the toxin release mechanism (Pei et al., 2020). Remarkably, Rhs-associated toxins commonly act on targets within the cytoplasm, with the most

common target predicted to be nucleic acids (Zhang et al., 2012). This in turn suggests that Rhs core could provide a mechanism for the effectors to reach the cytoplasm. Rhs undergoes complex genetic rearrangements to diversify the C-terminal sequences (Poole et al., 2011). New Rhs toxins could be acquired by HGT via recombination at conserved core regions (Jackson et al., 2009; Koskiniemi et al., 2014).

5 | CONCLUDING REMARKS AND FUTURE DIRECTIONS

5.1 | Discovering the myriad of the effectors

Bacteria can encode multiple T6SS loci and a plethora of effectors. The disposition of different toxic effector-immunity pairs, as well as cheating by the collection of the stand-alone immunities, helps both hide and seek in the dense and diverse communities (Barretto & Fowler, 2020; Kirchberger et al., 2017; Ross et al., 2019; Zhang et al., 2012). Highly diverse effector repertoire continues to be uncovered in different clinical and environmental isolates. Comparative genomic strategies combined with genetic screening help to identify novel T6SS effectors and their targets (Fridman et al., 2020). We are only beginning to learn about the acquisition and integration of novel effectors into the T6SS pathway. Bioinformatics studies suggest that inherent conserved regions of the effector recruitment domains such as Rhs, DUF4123 or MIX have diversifying capacity as they are prone to genetic rearrangements (Koskiniemi et al., 2014; Salomon, 2016; Unterweger et al., 2015). However, only a few genetic studies have demonstrated the engagement of distantly located so-called orphan-toxins (Koskiniemi et al., 2014; Ma et al., 2017a). Other polymorphic toxic systems that were found to share some toxic domains with T6SS are considered to be an alternative reservoir for novel toxic activities (Salomon, 2016; Zhang et al., 2012). The co-regulation of the T6SS expression with the DNA uptake could provide genetic material for the acquisition of novel effectors (Borgeaud et al., 2015; Ringel et al., 2017). Nevertheless, further research is needed to gain the full picture of the evolution of new effectors.

5.2 | Variations and limitations of T6SS-based delivery

In theory, some T6SS assemblies could accommodate several different effectors at once, leading to the secretion of a cocktail of effectors in one T6SS shot. Moreover, the same VgrG spike can accommodate different effectors using different PAAR or adaptor proteins, and some effectors could be recruited to several different assemblies (Burkinshaw et al., 2018; Cianfanelli et al., 2016a; Whitney et al., 2014; Wood et al., 2019b). Processes that could co-ordinate the secretion of different effectors and the bases for the selection of the effectors are so far unclear. The first high-resolution Cryo-EM structures of T6SS secretion spike/effector complexes revealed networks of highly specific interactions (Flaugnatti et al., 2020; Quentin et al., 2018). These examples represent only a few out of many possible secretion complex architectures. Moreover, to date, we lack the understanding of how the spike/effector complex accommodates in the baseplate and passes across the membrane complex. High-resolution structures of different architectures and different steps of assembly are needed to understand the molecular determinants of this process. This information is crucial in order to design inhibitors that could block the T6SS-based translocation of clinically important effectors. Moreover, first attempts to use the T6SS for delivery of heterologous proteins were successful but revealed some important limitations likely related to steric hindrances (Ho et al., 2017; Ma et al., 2009; Wettstadt et al., 2019; Wettstadt et al., 2020). Therefore, a better understanding of effector translocation at the molecular level could help harness T6SS for therapeutic interests, such as the injection of antibodies or antibiotics directly into the target cells.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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