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SUMMARY

The bacterial type VI secretion system (T6SS) system is a contractile secretion apparatus that delivers proteins to neighbouring bacterial or eukaryotic cells. Antibacterial effectors are mostly toxins that inhibit 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 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.

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 (Zoued *et al.*, 2013, Cherrak *et al.*, 2018). 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 (Leiman *et al.*, 2009, Basler *et al.*, 2012, 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 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 (Pukatzki *et al.*, 2007, Shneider *et al.*, 2013, Bondage *et al.*, 2016, Unterweger *et al.*, 2017, Ma *et al.*, 2017a). 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 (Durand *et al.*, 2014, Russell *et al.*, 2014, Cianfanelli *et al.*, 2016b, Hachani *et al.*, 2016). To prevent elimination of

kin cells, the antibacterial T6SS toxin activity is counteracted by cognate immunity proteins acting as anti-toxins. The acquisition and diversification of the effectors is associated with horizontal gene transfer, gene duplications and recombination through conserved sequences that locate new toxins and immunities at specific locations in the genome (Unterweger *et al.*, 2015, Salomon, 2016, Koskiniemi *et al.*, 2014). The arsenal of effectors and the stockpile of immunities discussed in this review is key for survival in the ecological niches with intense competition.

ACTIVITY OF T6SS DELIVERED EFFECTORS

In general, the activity of T6SS antibacterial effectors can be subdivided to 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 (Wang *et al.*, 2015, Si *et al.*, 2017a, Si *et al.*, 2017b, Han *et al.*, 2019). 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 (Zhang *et al.*, 2012, Smith *et al.*, 2020). 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, Ringel *et al.*, 2017, Lin *et al.*, 2019). 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+ (Alcoforado Diniz & Coulthurst, 2015, Whitney *et al.*, 2015, Ahmad *et al.*, 2019).

Periplasmic acting effectors

Phospholipases. Type VI lipase effectors (Tle) cause cell lysis of competing bacteria by hydrolyzing phospholipids (Russell *et al.*, 2013, Jiang *et al.*, 2014, Ma *et al.*, 2014a, Jiang *et al.*, 2016, Flaugnatti *et al.*, 2016). 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 (Russell *et al.*, 2013, Hu *et al.*, 2014, Jiang *et al.*, 2016, Flaugnatti *et al.*, 2016). 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 (Hu *et al.*, 2014, Flaugnatti *et al.*, 2016, Jiang *et al.*, 2016, Berni *et al.*, 2019, 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 (Russell *et al.*, 2013, Jiang *et al.*, 2014, 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 accumulation of phosphatidic acid generated by cleavage of the head groups of phospholipids (Russell *et al.*, 2013, Lery *et al.*, 2014). 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 activation of the PI3 kinase pathway, while TplE/Tle4 activity leads to endoplasmic reticulum disruption, triggering autophagy (Jiang *et al.*, 2016, Jiang *et al.*, 2014).

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 (Miyata *et al.*, 2013, LaCourse *et al.*, 2018, Mariano *et al.*, 2019, Fridman *et al.*, 2020). These effectors share homologies with pore-forming colicins or have predicted transmembrane helices (TMH) in their C-terminal region (Miyata *et al.*, 2011, LaCourse *et al.*, 2018, Fridman *et al.*, 2020). 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 (Miyata *et al.*, 2013, Fridman *et al.*, 2020). However, the precise molecular mechanisms underlying pore formation and/or membrane disruption induced by these different toxins remain to be determined.

Peptidoglycan hydrolases. A broad spectrum of T6SS effectors cause 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, Russell *et al.*, 2011, Whitney *et al.*, 2013, Ma *et al.*, 2018). *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 (Dong *et al.*, 2013b, Brooks *et al.*, 2013). This is reminiscent of the VgrG homologue 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 a 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).

Metallopeptidases. Periplasmic-acting Type VI peptidase effectors (Tpe) belong to zinc dependent metallopeptidases (Peptidase_MA, CL0126) that bear a HExxH zinc-binding motif (Zhang *et al.*, 2012, Wood *et al.*, 2019a). *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 (Zhang *et al.*, 2012, Ma *et al.*, 2017b).

Cytoplasmic acting effectors

DNases. Most of the T6SS DNase effector (Tde) toxins belong to HNH superfamily, also known as His-Me finger endonucleases (Zhang *et al.*, 2012, Koskiniemi *et al.*, 2013, Alcoforado Diniz & Coulthurst, 2015, Pissaridou *et al.*, 2018, Pei *et al.*, 2020). 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 induction of the SOS response and eventually lose DAPI staining indicating complete degradation of chromosomal DNA (Pissaridou *et al.*, 2018, Ma *et al.*, 2017a, Jana *et al.*, 2019). 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 (Ma *et al.*, 2014b, Bondage *et al.*, 2016, Bernal *et al.*, 2017, Zhang *et al.*, 2012, Jana *et al.*, 2019). 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).

***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 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).

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 (Whitney *et al.*, 2015, Tang *et al.*, 2018). Drastically reduced NAD(P) + concentrations in the intoxicated cell lead to general growth inhibition with cells maintaining structural integrity (Whitney *et al.*, 2015).

(p)ppApp synthetases. Tas1 effector secreted by a virulent clinical isolate of *P. aeruginosa* structurally resembles RSH domain (RelA-SpoT homologue) 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).

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).

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 (Wang *et al.*, 2015, Si *et al.*, 2017a, Si *et al.*, 2017b, Han *et al.*, 2019, DeShazer, 2019). *P. aeruginosa* secreted TseF effector doesn't 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 (Si *et al.*, 2017a, Si *et al.*, 2017b, Lin *et al.*, 2017).

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 (Russell *et al.*, 2013, Flaugnatti *et al.*, 2016, Fridman *et al.*, 2020, 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 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 (Zhang *et al.*, 2012, Zhang *et al.*, 2013a, Zhang *et al.*, 2013b, Dong *et al.*, 2013a, Ding *et al.*, 2012, Wang *et al.*, 2013, Whitney *et al.*, 2013, Robb *et al.*, 2016), 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 (Zhang *et al.*, 2012, Kirchberger *et al.*, 2017, Ross *et al.*, 2019, Barretto & Fowler, 2020). The

acquisition of new immunity genes is associated with horizontal gene transfer and recombination (Zhang *et al.*, 2012, Kirchberger *et al.*, 2017).

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 (Unterweger *et al.*, 2015, Unterweger *et al.*, 2017, Shneider *et al.*, 2013, Silverman *et al.*, 2013, Ma *et al.*, 2017a) (Figure 3).

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) (Uchida *et al.*, 2014, Spinola-Amilibia *et al.*, 2016). The gp5 needle domain can be followed by additional effector domains or effector-recruitment domains (Pukatzki *et al.*, 2007, Flaugnatti *et al.*, 2016). PAAR domain proteins form cone shaped structures (Shneider *et al.*, 2013, Rigard *et al.*, 2016). 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 (Shneider *et al.*, 2013, Rigard *et al.*, 2016). 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 chaperone functions (Silverman *et al.*, 2013).

Different effector groups may prefer certain types of core component carrier. 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 (Liang *et al.*,

2015, Unterweger *et al.*, 2015, Flaugnatti *et al.*, 2016, Wettstadt *et al.*, 2019, Ma *et al.*, 2020, Flaugnatti *et al.*, 2020). Nevertheless, some Tle effectors fused to PAAR or Hcp were identified and await characterization (Ma *et al.*, 2017a, Flaugnatti *et al.*, 2020). On the contrary, cytoplasmic acting toxins are almost exclusively fused to PAAR, Hcp or specialized recruitment domains (Ma *et al.*, 2017a, Koskiniemi *et al.*, 2013, Pissaridou *et al.*, 2018, Bernal *et al.*, 2017, Jana *et al.*, 2019, Tang *et al.*, 2018, Ting *et al.*, 2018, Ahmad *et al.*, 2019, Mok *et al.*, 2020). Structural motifs encoded in these domains might facilitate translocation of these toxins to the cytoplasm of the prey.

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 secretion of their cognate effector, although not secreted themselves (Bondage *et al.*, 2016, Unterweger *et al.*, 2015, Cianfanelli *et al.*, 2016a, 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 (Whitney *et al.*, 2015, Cianfanelli *et al.*, 2016a, Quentin *et al.*, 2018). 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 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 (Unterweger *et al.*, 2015, Liang *et al.*, 2015, Bondage *et al.*, 2016, Burkinshaw *et al.*, 2018). 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 (Unterweger *et al.*, 2015, Liang *et al.*, 2015). These chaperones are encoded upstream their cognate effectors and have C-terminal ends specific to the N-terminal domains of their cognate effectors (Unterweger *et al.*, 2015, Liang *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).

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 (Salomon *et al.*, 2014, Jana *et al.*, 2019). 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 to 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 (Shneider *et al.*, 2013, Flaugnatti *et al.*, 2016,

346 Wood *et al.*, 2019b, Wettstadt *et al.*, 2019, Flaugnatti *et al.*, 2020). Bacterial TTR-like domains are
347 globular structures composed of two small β-sheets structurally resembling the eukaryotic TTR β-
348 sandwich structure (Flaugnatti *et al.*, 2020). In enteroaggregative *E. coli* $VgrG^{EAEC}$, the TTR is fused
349 to the VgrG gp5 needle-like domain through highly flexible loops (Flaugnatti *et al.*, 2020). TTR
350 interacts with the flexible N-terminal extension of Tle1 effector which inserts its β-sheets to form a
351 β-barrel with TTR (Flaugnatti *et al.*, 2020). In addition to recruitment function, TTRs can also provide
352 stability to the effectors as well as participate in effector neutralization (Wettstadt *et al.*, 2019,
353 Flaugnatti *et al.*, 2020). DUF2345 domain forms an extension of VgrG by continuing a β-prism
354 structure of the C-terminal gp5 domain of the VgrG spike (Flaugnatti *et al.*, 2020). DUF2345, as well
355 as long α-helices aligned against the length of the needle, make numerous contacts with loaded
356 effector. Together with the TTR domain, they can be considered an internal adaptor domain for direct
357 recruitment of the effectors (Flaugnatti *et al.*, 2020). DUF2345 domains can perform additional
358 functions. In *Acinetobacter baumannii*, the DUF2345 of VgrG is essential for T6SS assembly, while
359 the VgrG4 DUF2345 domain of *Klebsiella pneumoniae* is itself an antibacterial and antifungal toxic
360 effector inducing response to reactive oxygen species (Lopez *et al.*, 2020, Storey *et al.*, 2020). Rhs
361 are large proteins that were first identified as recombination-prone loci in chromosomes (Lin *et al.*,
362 1984). Certain classes of Rhs proteins are secreted through T6SS (Koskiniemi *et al.*, 2013,
363 Alcoforado Diniz & Coulthurst, 2015, Pei *et al.*, 2020). They are encoded downstream of VgrG or
364 T6SS chaperones (Eag or Tec/Tap) and comprise N-terminal PAAR or other VgrG interacting
365 (VIRN) domains (Cianfanelli *et al.*, 2016a, Pei *et al.*, 2020). The Rhs core region comprised of
366 spiralling β-sheets forms a shell-like structure that likely encloses the C-terminal toxin domain
367 (Jackson *et al.*, 2009, Zhang *et al.*, 2012, Alcoforado Diniz & Coulthurst, 2015, Jackson *et al.*, 2019).
368 It has been recently demonstrated that a T6SS secreted Rhs protein undergoes two autocleavages –
369 one at the N-terminus and one at the C-terminus (Pei *et al.*, 2020). These conserved autocleavages
370 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).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

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 collection of the stand-alone immunities helps both hide and seek in the dense and diverse communities (Zhang *et al.*, 2012, Kirchberger *et al.*, 2017, Ross *et al.*, 2019, Barretto & Fowler, 2020). Highly diverse effector repertoire continues to be uncovered in different clinical and environmental isolates. Comparative genomic strategies combined with genetic screening helps identifying novel T6SS effectors and their targets (Fridman *et al.*, 2020). We are only beginning to learn about the acquisition and integration of the 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, Unterweger *et al.*, 2015, Salomon, 2016). 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 (Zhang *et al.*, 2012, Salomon, 2016). The co-regulation of the T6SS expression with the DNA uptake could provide genetic material for acquisition of novel effectors (Borgeaud *et al.*, 2015, Ringel *et al.*, 2017). Nevertheless, further research is needed to gain full picture of the evolution of new effectors.

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 (Whitney *et al.*, 2014, Cianfanelli *et al.*, 2016a, Burkinshaw *et al.*, 2018, Wood *et al.*, 2019b). Processes that could coordinate the secretion of different effectors and the bases for the selection of the effectors are so far unclear. First high-resolution Cryo-EM structures of T6SS secretion spike/effector complexes revealed networks of highly specific interactions (Quentin *et al.*, 2018, Flaugnatti *et al.*, 2020). These examples represent only a few out of many possible secretion complex architectures. Moreover, to date we lack understanding of how 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 T6SS based translocation of clinically important effectors. On the other hand, first attempts to use the T6SS for delivery of heterologous proteins were successful but revealed some important limitations likely related to steric hindrances (Ma *et al.*, 2009, Ho *et al.*, 2017, Wettstadt *et al.*, 2019, Wettstadt *et al.*, 2020). Therefore, better understanding of effector translocation at the molecular level could help harness T6SS for therapeutic interests, such as injection of antibodies or antibiotics directly into the target cells.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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FIGURE LEGENDS

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 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

761 secreted components are shown in dark blue – the tail tube consists of a stack of hexameric Hcp rings
762 and spike consists of VgrG and PAAR. The effectors are shown in red.
763
764 **Figure 2. Anti-bacterial toxic activities of T6SS effectors.** The scheme shows the mode of action
765 of the different families of T6SS toxins. All toxins are shown in red.
766 **Tle** degrade membrane phospholipids (in light blue). **Tae and Tge** effectors degrade the
767 peptidoglycan (in golden) through amidase or glycoside hydrolase activities respectively. **Tme** form
768 pores in the inner membrane, leading to membrane depolarization (ions in black). One member of the
769 **Tpe** family was proposed to hydrolyze the lipid anchor of outer membrane lipoproteins (in green).
770 **Tas1** phosphorylates ATP and ADP (shown in yellow) leading to accumulation of (p)ppApp (shown
771 in orange). **Tne** degrade NAD(P) + (shown in pink) to nicotinamide (brown) and ADP-ribose (dark
772 purple), leading to the depletion of NAD(P) + cellular pools. **Tde** catalyze non-specific hydrolysis of
773 DNA (shown in black). **DddA** deaminates cytosine to uracil in the double stranded DNA. DddA
774 caused mutations are depicted in red, ammonia released as reaction product in grey. **Tre** ADP-
775 ribosylates protein target (here FtsZ) upon NAD + hydrolysis. FtsZ proteins are shown in blue, NAD
776 + in pink, ADP-ribose in dark purple.
777
778 **Figure 3: Currently described modes of effector loading on VgrG, Hcp or PAAR.** Effectors
779 (shown in red) can be fused to Hcp, VgrG or PAAR components or be encoded separately and interact
780 with these components directly or with the help of chaperones/adaptors. Chaperones can be either
781 encoded separately (shown in green) or constitute recruitment motifs that are integral parts of VgrG
782 or PAAR. Chaperones (Eag, Tep/Tec), Rhs domain, as well as a TTR domain are indicated.
783

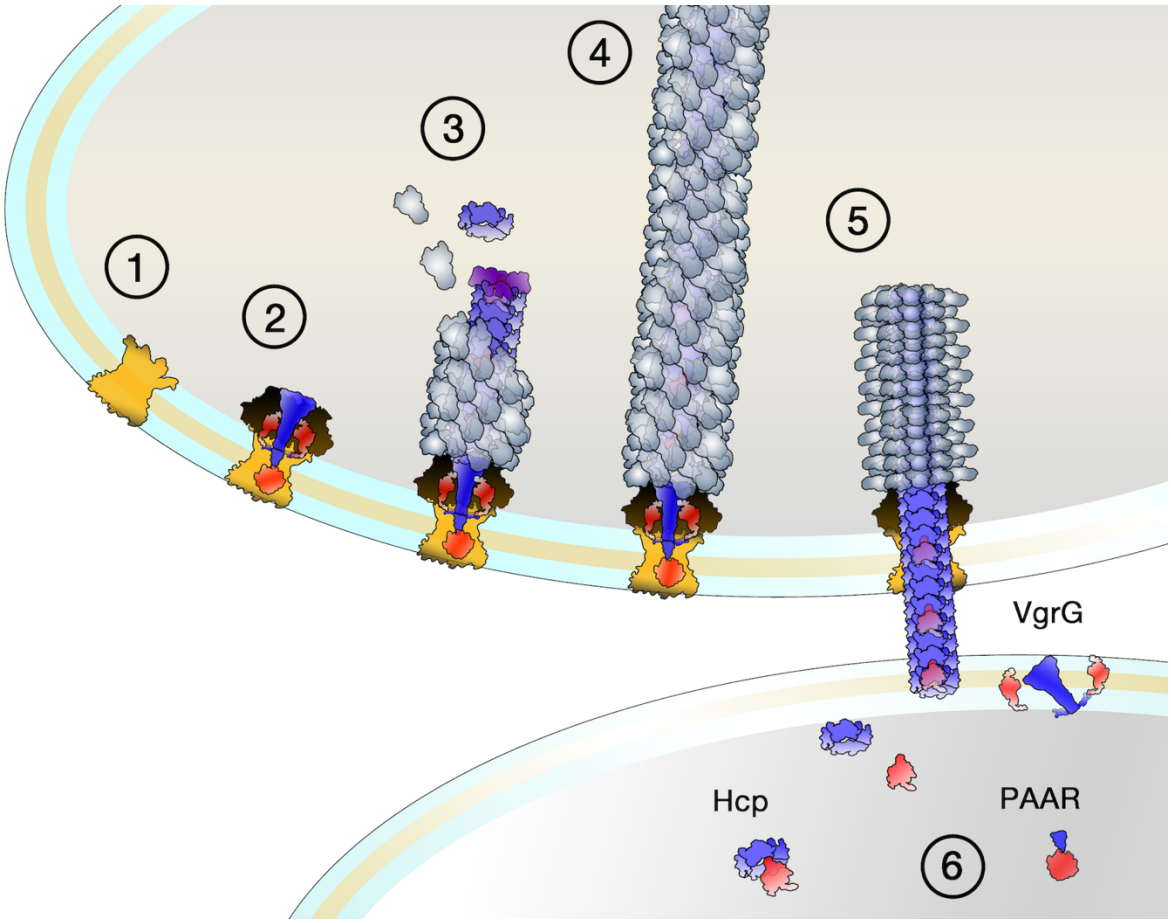
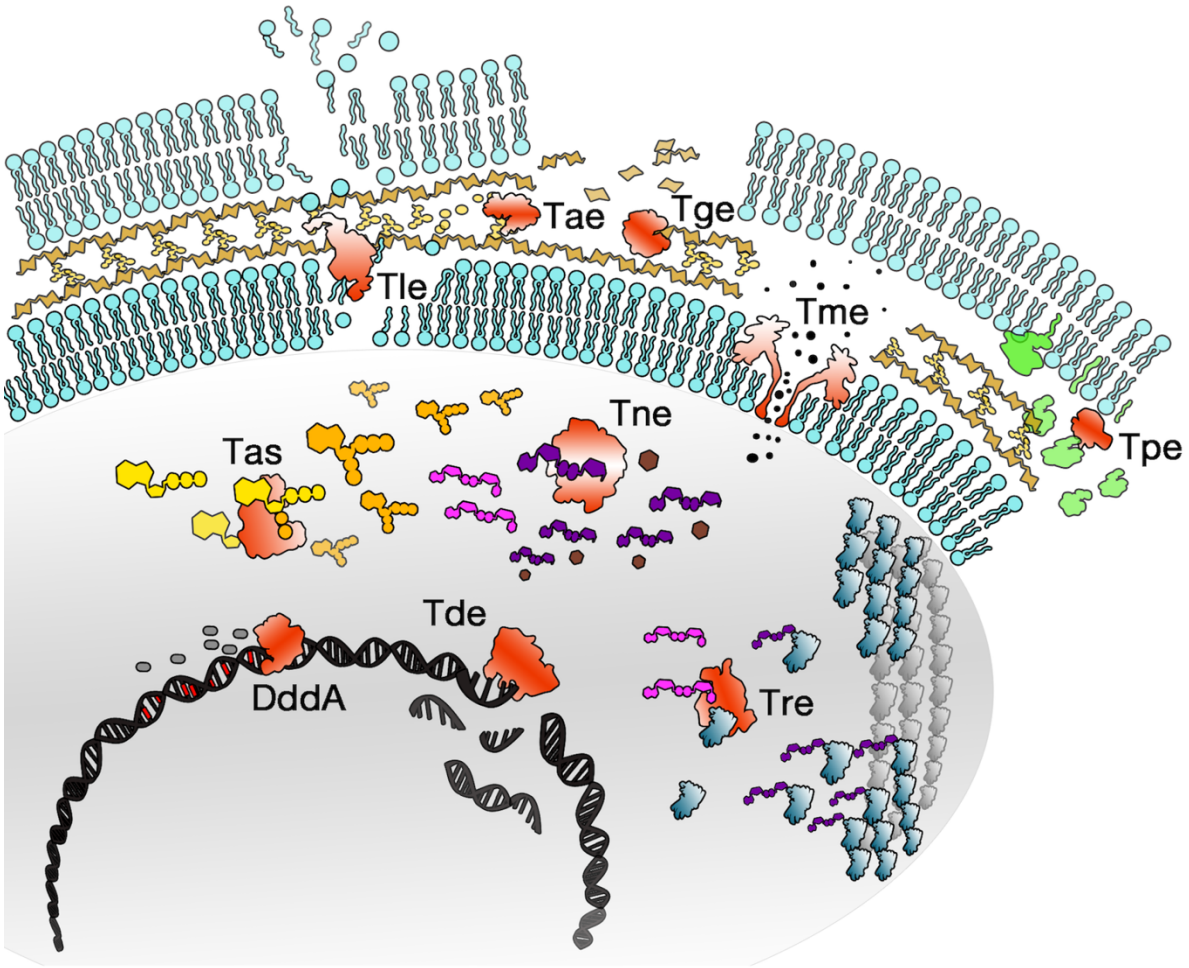


Figure 1



Periplasmic effectors

Tle - Type 6 Lipase Effector
Tae - Type 6 Amidase Effector
Tge - Type 6 Glycoside hydrolase Effector
Tme - Type 6 Membrane-disrupting Effector
Tpe - Type 6 Peptidase Effector

Cytoplasmic effectors

Tas - Type 6 Effector (p)ppApp Synthetase
Tne - Type 6 NADase Effector
Tde - Type 6 DNase Effector
DddA - DS-DNA Deaminase toxin A
Tre - Type 6 ADP-Ribosyltransferase Effector

Figure 2

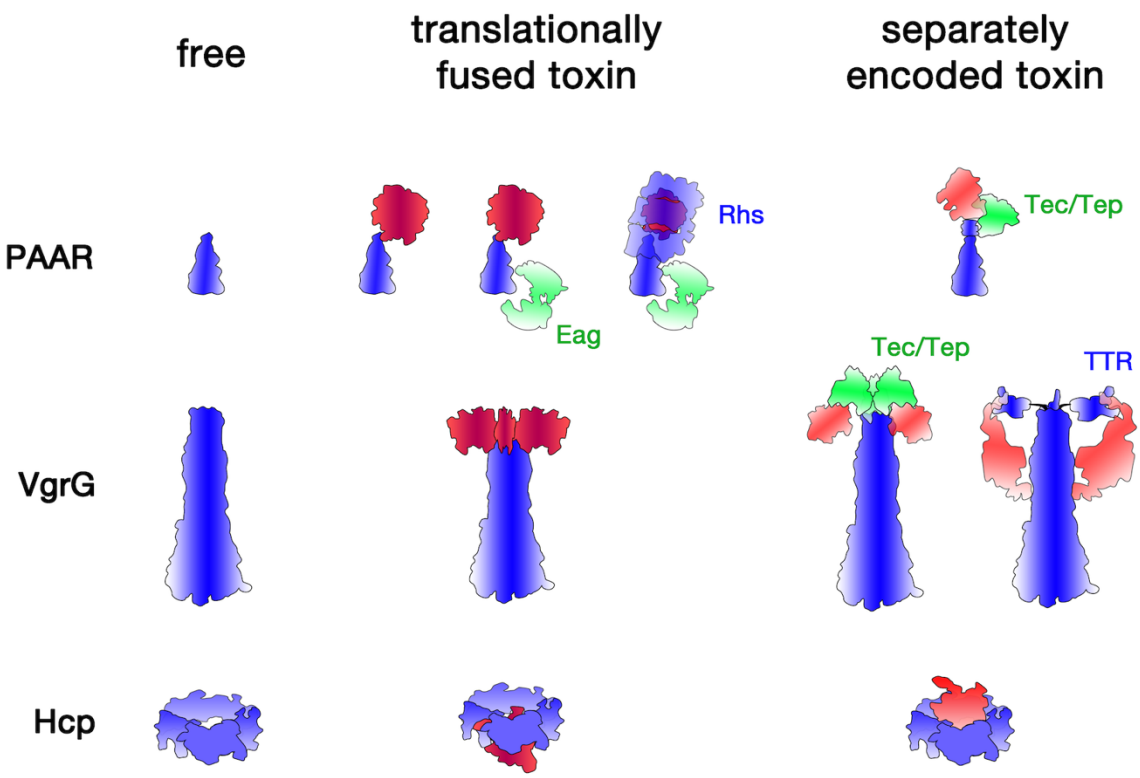

free
translationally fused toxin
separately encoded toxin
PAAR
VgrG
Hcp
Rhs
Eag
Tec/Tep
Tec/Tep
TTR

Figure 3