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Type VI secretion effectors: poisons with a purpose

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Abstract

The type VI secretion system (T6SS) mediates interactions between a diverse range of Gram-negative bacterial species. Recent studies have led to a drastic increase in the number of characterized T6SS effector proteins and produced a more complete and nuanced view of the adaptive significance of the system. While the system is most often implicated in antagonism, in this review we consider the case for its involvement in both antagonistic and non-antagonistic behaviors. Clarifying the roles that T6S plays in microbial communities will contribute to broader efforts to understand the importance of microbial interactions in maintaining human and environmental health, and will inform efforts to manipulate these interactions for therapeutic or environmental benefit.

Bacteria colonize nearly every imaginable habitat on earth. Many environments, ranging from soil to vertebrate digestive tracts, harbor a wide diversity of bacterial species[1,2]. In other habitats, such as in the light organ of the bobtail squid[3] and certain acute infections, single species of bacteria can dominate. However, even when bacterial species diversity is low, individual bacterial cells rarely live in isolation. Instead, they typically grow, divide and die in close proximity to other bacterial cells. Accordingly, every aspect of bacterial growth and physiology has the potential to be influenced by interbacterial interactions. New mechanisms by which bacteria interact continue to be discovered, and range from simple competition for nutrients, to highly evolved symbioses, as in the formation of metabolically-dependent structured consortia[3–6]. Evidence from a variety of habitats now suggests that the outcome of bacterial interactions can have profound consequences for ecosystem function, as well as for human health[7–9].

Recently, it was discovered that one mechanism by which Gram-negative cells in close proximity can interact is through contact-dependent transport of proteins from a donor cell to a recipient cell via the activity of an apparatus known as the type VI secretion system (T6SS) (Box 1). This system was initially found to deliver effector proteins to eukaryotic cells, however it has since been shown to more often mediate interbacterial interactions (Box 2)[10]. Typically encoded by clusters of contiguous genes, the T6SS is a complex structure composed of 13 conserved proteins and a variable complement of accessory elements. T6SSs are widely distributed in the genomes of Proteobacteria with some species encoding as many as six phylogenetically and functionally divergent systems[11–13]. T6SS

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gene clusters are found in free-living and eukaryote-associated species, including both pathogens and symbionts of animals and plants[11].

Box 1

Structure and function of the T6SS

The T6SS is thought to consist of two main complexes in association with additional bridging and cytoplasmic elements: a membrane-associated assembly, which includes two proteins homologous to elements of bacterial type IV secretion systems, and an assembly with components that bear a structural resemblance to bacteriophage sheath, tube and tail spike proteins[127–129]. These two subassemblies work together by an unknown mechanism to translocate effector proteins across the envelope of the donor cell, and then through the outer membrane of a recipient cell. While the superstructure of the T6SS remains unsolved, analyses of the individual components have produced a theoretical model by which the system might function (reviewed in [130,131]). Current hypotheses concerning the function of the T6SS focus predominantly on the constituents of a phage-like subassembly, as most available structure–function data concerns this putative complex. The phage-like elements comprising an active T6SS (and their phage analogs) are: TssB and TssC (bacteriophage contractile sheathe), Hcp (gp19 tail-tube protein), TssE (gp25 baseplate assembly protein), and VgrG (a fusion of the gp5-gp27 tip proteins)[127,128]. By analogy with their phage counterparts, these components of the T6SS are thought to resemble an inverted bacteriophage, with VgrG forming a cell-puncturing tip, Hcp forming a tail-tube structure through which effector proteins might travel, and TssB and TssC forming a sheathe which contracts to provide energy for effector translocation. Notably, while a dynamic TssB/C sheathe has been directly observed, the remainder of the inverted-phage hypothesis still requires *in vivo* confirmation[129].

An additional complexity of the structure of the T6SS is that Hcp and VgrG are not only essential components of the system, but are also shed into the extracellular milleux upon activation of the system – indicating that these proteins occupy a dual role as both structural components and substrates of the T6SS[130]. Moreover, while most of the thirteen core T6SS genes are found in single copy within a given secretion locus, multiple Hcp and VgrG homologs are often associated with and secreted by a single T6SS[14,44,132]. This leads to the hypothesis that Hcp and VgrG proteins are function as adaptors that interact with, and recruit, effector proteins to the apparatus. This has been supported by the presence of *vgrG* and *hcp* genes in operons containing T6S effectors, the observation that effector fusions can occur to both Hcp and VgrG, the finding that effector-linked PAAR domains can directly interact with VgrG, and, most directly, by the finding that allele-specific interactions with the pore of Hcp is required for the stabilization and secretion of certain effectors[17,19,35,106,133,134].

Box 2

The T6SS before "T6"

What we now refer to as the T6SS was discovered independently by two groups as a secretion system before the term "type VI secretion system" was coined[135]. The system was first described as a cluster of impaired in nitrogen fixation (*imp*) genes in *Rhizobium leguminosarum*, wherein mutations in several *imp* genes, now known to encode core components of the secretory apparatus, were shown to influence the symbiotic host range of the organism[136]. Further noted was the conservation of *imp*-like gene clusters in assorted bacteria, that certain Imp proteins bore similarity to those involved in characterized secretion pathways, and that *imp* mutants are defective in protein secretion. Remarkably, in reference to the phenotypes observed for *imp* mutations, Spaink and colleagues concluded they were "caused by genes that are most likely involved in the temperature-dependent secretion of proteins". Temperature-dependent T6S activation has since been observed by several groups and is now a well-studied phenomenon[47,57,59,60].

Shortly after the groundbreaking work on the *imp* genes of *R. leguminosarum*, Leung and co-workers identified the T6SS of *Edwardsiella tarda* in a mass spectrometric screen for secreted virulence factors[137]. The authors identified two secreted proteins, which they termed *E. tarda* virulence proteins A and C (EvpA and EvpC), and mapped the open reading frames encoding these proteins to a large conserved gene cluster, *evpA-H*, that is now recognized as a T6SS[138]. Based on the requirement they observed for *evpA* and *evpB* in the export of EvpC – an Hcp homolog – the authors accurately concluded that Evp encodes a novel protein secretion system.

The first indication that T6S could be involved in interbacterial interactions came through the identification of three effector proteins secreted by the haemolysin co-regulated protein (Hcp) secretion island-I-encoded T6SS of *Pseudomonas aeruginosa* (H1-T6SS)[14]. Each of these effectors exhibits toxicity toward bacteria and is encoded adjacent to a gene whose product provides immunity to the toxin, thereby preventing self-intoxication[15]. Growth competition assays between a donor strain capable of toxin secretion and a recipient strain engineered to lack one or more effector–immunity (E–I) gene pairs demonstrated that effectors are translocated between bacteria via the T6SS, and that this process confers a significant fitness advantage to donor strains. As the T6SSs of additional bacteria are studied, and their effectors identified, it is becoming evident that the delivery of toxic effectors to other bacterial cells is a fundamental activity of the system.

Although we now know that many bacterial species, including the pathogenic organisms *Serratia marcescens*, *Vibrio cholerae*, and *P. aeruginosa*, are equipped to deliver effector proteins to other bacteria via T6S under laboratory conditions, the significance of this activity in natural communities remains unclear[14,16,17]. While the toxicity of the bacterial-targeted effectors identified to date suggests that T6S is important for bacterial competition, it is not known to what extent toxin translocation facilitates invasion of bacteria into new habitats, or protects established populations from invasion by incoming competitors. It is also not understood to what extent T6SS-mediated antagonism facilitates competition

between individual bacteria within the same species versus between species. As elaborated upon below, there are also indications that the function of T6S can extend beyond simple antagonism. Clarifying the role that the system plays in a natural setting will further our understanding of the mechanisms that shape microbial community structure, and the resulting consequences for ecosystem function. In this review we consider the myriad functions T6S might serve in mediating interactions between diverse bacteria, focusing on those possibilities suggested by the activities of known interbacterial effector proteins.

Effector proteins of the T6SS

T6SS effectors come in many forms, ranging from relatively simple single-domain proteins to large multi-domain proteins composed of apparent fusions between toxins and secreted structural elements of the T6SS[14,18]. In spite of this diversity, common themes that likely reflect highly conserved mechanistic aspects of the T6 interbacterial targeting system have emerged. Perhaps most pervasive is the co-occurrence of effectors with cognate immunity proteins[19]. These proteins are generally encoded adjacent to cognate effector genes and effector inactivation is achieved by a direct binding mechanism[20,21]. As the majority of immunity proteins are required only for defense against effectors delivered by neighboring cells, this co-occurrence underscores the capacity of the T6SS to attack both friend and foe.

Cell wall-degrading effectors

As the major structural component of the bacterial cell wall, peptidoglycan (PG) is a preferred target of antibacterials. This is perhaps most convincingly shown by the convergent evolution of numerous inter-organismal competitive strategies that target PG [22–26]. It is therefore not surprising that enzymes acting on PG are a major component of the T6S effector arsenal (Figure 1, panel 1). Indeed, Tse1 and Tse3 (type VI secretion exported 1 and 3), the first antibacterial T6S effectors to be characterized biochemically, both exhibit PG-degrading activity[15]. By targeting the peptide and glycan moieties of the molecule, respectively, these enzymes have the potential to digest the macromolecular sacculus of a typical Gram-negative bacterium into small, soluble fragments.

P. aeruginosa Tse1 is arguably the most thoroughly characterized T6S effector. Soon after its discovery and characterization, several groups reported X-ray crystal structures which showed that, like certain housekeeping amidases, Tse1 is a compact molecule that adopts a papain-like cysteine protease fold[20,27–29]. However, structural studies have so far fallen short of providing an explanation for its context-specific activity. PG consists of a glycan backbone composed of alternating β1,4-linked *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc) subunits. The MurNAc residues are further modified with peptides that can vary both in length and composition which provide structural support for the molecule by engaging in crosslinks to adjacent strands. PG crosslinks are asymmetric, occurring between the fourth amino acid (D-Ala) of the acyl donor peptide and the third amino acid (*meso*-diaminopimelic acid) of the acceptor peptide. Interestingly, Tse1 cleaves between the second and third residues of the peptide stem, yet it exhibits a strong preference for this site within pentapeptide strands or for the donor strand within tetrapeptide crosslinks[15,27]. Given that PG is first incorporated into the sacculus in the pentapeptide form, this specificity could direct Tse1 to sites of PG synthesis, the disruption of which has

catastrophic effects on the cell[24–26]. In support of this hypothesis is the observation that Tse1 is responsible for the majority of H1-T6SS-dependent lysis of competing organisms[30].

Tse1 belongs to a larger group of T6S effectors, referred to as the Tae (type VI amidase effector) superfamily[19]. Tae proteins comprise at least four highly divergent families (Figure 1, panel 1). While the evolutionary relationship between these groups has not yet been ascertained, all available experimental evidence indicates the Tae proteins are functional analogs. For instance, *tae* genes are invariably located adjacent to open reading frames encoding periplasmic immunity determinants, termed Tai (type VI secretion amidase immunity) proteins. Further uniting the Tae superfamily is the observation that each of its members so far characterized – including representatives from each family – function as amidases that catalyze the hydrolysis of Gram-negative PG. The activity of only a few Tae proteins has been examined in interbacterial competition, however the consensus reached from studies examining Tae1,2 and 4 members is that the proteins also display a common reliance on the T6SS to act effectively on target cells[16,19]. As Tae proteins are exported by multiple T6S clades within a diversity of T6SS-positive organisms, the shared properties of the Tae superfamily suggest a significant extent of functional and mechanistic conservation throughout T6S.

As mentioned, Tse3 acts on the glycan backbone of PG rather than the peptide crosslinks. The β1,4 bonds between MurNAc and GlcNAc and GlcNAc and MurNAc are both targets of antimicrobial enzymes, and cleavage of either bond by muramidases and glucosaminidases, respectively, has the potential to impair the integrity of the PG sacculus[31]. While Tse3, a T6S glycoside hydrolase with characterized cleavage site specificity, acts as muramidase, this protein, belongs to a larger superfamily of T6S glycoside hydrolase effectors (Tge) that may additionally include glucosaminidase enzymes[15,32] (Figure 1, panel 1). Three sequence-divergent groupings of Tge proteins have been identified (Tge1–3), with the first (Tge1) composed solely of Tse3. Though the Tge3 family has yet to be experimentally validated, a Tge2 family member from *Pseudomonas protegens* ($Tge2^{PP}$) was shown to transit the T6S pathway and serve as an antibacterial effector. The bond cleavage specificity of $Tge2^{PP}$ has not been determined, however the X-ray crystal structure of the protein places it in glycoside hydrolase family 73. These enzymes share the lysozyme muramidase fold, but are distinguished by a conserved active site tyrosine residue and generally exhibit *N*-acetylglucosaminidase activity.

Beyond its posited structural role in the T6S apparatus, the VgrG protein appears to directly facilitate the translocation of certain effectors and effector domains. The C-terminal effector domain of *V. cholerae* VgrG3 has the predicted fold of a muramidase, causes lysis when directed to the periplasm and can degrade PG (Figure 1, panel 1)[18]. Furthermore, the presence of a *vgrG3* cognate immunity gene is essential only in strains containing an active T6SS, indicating that the VgrG3 protein participates in T6S-mediated intercellular competition[17]. Given that the VgrG3 effector domain is unrelated to Tge family proteins and is likely exported by a distinct mechanism, it appears to represent a distinct family of muramidase effectors. VgrG3 also provides the first example of a VgrG effector domain that participates in interbacterial T6S, as all previously characterized VgrG effector domains were found to mediate microbe-host interactions[33,34]. The finding that effectors belonging to

the same catalytic class and functional in the same compartment of the cell can exist both as fusion proteins and independent effectors has important implications. It suggests that the effector domains of specialized VgrG proteins can derive from pre-existing effectors and that the evolutionary pressure driving the formation of VgrG–effector fusions is not necessarily one related to unique delivery requirements or target organisms. Instead, the selective pressure driving VgrG–effector fusions may be more commonplace and be related to achieving efficient co-regulation and accurate stoichiometry.

Cell membrane-targeting

The cell membrane, like the cell wall, is a conserved and essential component of the bacterial cell. It is therefore not surprising that this structure is also a target of T6S effectors (Figure 1, panel 2). A group of T6SS phospholipase effectors, the Tle (type VI lipase effector) proteins, directly target the bacterial membrane by hydrolyzing its component lipids[35]. There are five known Tle families, Tle1–5, that further distribute into two catalytic classes, those that appear to utilize a nucleophilic serine in a GxSxG motif (Tle1–4), and those that utilize dual catalytic histidine residues in HxKxxxxD motifs (Tle5). The Tle immunity proteins, Tli1–5 (type VI lipase immunity 1–5), localize to the periplasmic space and inactivate their cognate effectors directly, indicating that Tle effectors are likely to initiate membrane destruction from the periplasm, similar to cell wall-degrading effector proteins. Two Tle families have not been confirmed beyond their informatic identification, whereas representatives from Tle1, Tle2, and Tle5 have been both biochemically and phenotypically validated. Interestingly, these families degrade membranes by attacking different bonds in phospholipids (Figure 1, panel 2). In addition to displaying bond specificity within a given phospholipid, Tle proteins also appear to exhibit phospholipid headgroup preference. Phospholipid analysis of cells intoxicated with Tle5 from *P. aeruginosa* demonstrated that this phospholipase D enzyme exhibits a preference for phosphatidylethanolamine, the major phospholipid constituent of the bacterial membrane[35].

The intact structure of PG, and even the majority of its subunit constituents (eg. D-amino acids and MurNAc), are strictly bacterially associated, leaving little doubt that cell wall-targeting effectors act exclusively against bacterial targets. On the other hand, the membranes of bacteria and eukaryotes both consist primarily of phospholipids, with certain constituents differing only in their relative concentrations between the two types of organisms. This fact raises the intriguing possibility that the Tle effectors could participate both in interbacterial interactions, and, perhaps opportunistically, in mediating interactions with eukaryotic cells. In support of this notion, disruption of *P. aeruginosa tle5* and *V. cholerae tle2* attenuates these organisms in eukaryotic infection models[17,36]. The potential of Tle-based interdomain targeting by the T6SS will require additional study; however, it is worth noting that the invariable association of Tle effectors and Tli immunity proteins suggests the basal role of these proteins lies in interbacterial interactions.

The barrier provided by the cellular membrane is not only susceptible to hydrolysis by phospholipases, but can also be disrupted by the insertion of pore-forming proteins, which create channels that dissipate the chemiosmotic gradient. This strategy is utilized by bacteria that secrete bacteriocins, and has been well-studied for colicin Ia[37]. An antibacterial T6SS

effector in *V. cholerae*, VasX, and a protein secreted by an interbacterial T6SS in *B. thailandensis*, BTH_I2691, both have predicted structures that closely match that of colicin Ia[17,19,38,39] (Figure 1, panel 2). Additionally, open reading frames adjacent to *vasX* and BTH_I2691 encode predicted polytopic inner-membrane proteins, which mirror the topology and localization of immunity proteins to pore-forming bacteriocins[37,40]. Therefore, it appears likely that these two proteins and their homologs are pore-forming T6SS effectors – an additional strategy of targeting the bacterial membrane by T6S.

Nucleic acid-targeting

The cell envelope does not appear to contain all targets of the vast T6S effector repertoire. Nucleases, and predicted nucleases, are represented in the T6S interbacterial effector arsenal (Figure 1, panel 3). Recent studies of Rhs (recombination hot spot) elements suggest that a subset of these proteins are also T6SS nuclease effectors[42,43]. *Dickeya dadantii* Rhs proteins, RhsA and RhsB, contain C-terminal endonuclease-related effector domains and are transferred between bacterial cells in a manner dependent upon closely linked *vgrG* genes. Expressed in *E. coli*, these domains result in chromosomal and plasmid DNA degradation, and growth inhibition and a loss of DAPI staining accompanies their delivery to target cells. T6-dependent export of an Rhs protein in *S. marcescens* suggests this type of effector may play a broad role in T6S-mediated bacterial interactions[44]. Whether Rhs proteins and previously described T6S effector classes share the same genetic requirements for export, or whether Rhs proteins might usurp components of the T6S pathway to achieve intercellular transfer by a distinct mechanism remains to be determined.

The general study of both the form and function of effector proteins has greatly informed our understanding of interbacterial T6S. This increased knowledge of the theoretical capabilities of the T6SS has provided a framework for a sophisticated understanding of this pathway. With this in mind, in the remainder of this review we further explore the role of the T6SS in interbacterial competition, and, moreover, the potential of this system to mediate interactions above and beyond simple antagonism.

The case for interbacterial antagonism mediated by the T6SS

The role of T6S in mediating interbacterial antagonism has received significant attention. Systems in *Burkholderia thailandensis, P. aeruginosa*, *V. cholerae*, *V. parahaemolyticus, S. marcescens*, *Citrobacter rodentium*, *P. syringae*, and *Acinetobacter baumanii* have been demonstrated to provide increased fitness when these organisms are grown in competition with other bacteria in the laboratory[13,14,45–50]. Interbacterial T6S appears to allow bacteria to attack one another as they compete for space and resources. It is important to note, however, that bacteria live in dynamic communities often consisting of many species in close-association with each other and with additional biotic and abiotic elements. As it is within these complex surroundings that T6S evolved, it is difficult based solely on laboratory competition experiments to define with confidence the adaptive significance of the pathway. Nevertheless, in combination with growth competition studies, the results of biochemical, genomic, and regulatory analyses have – as outlined below – produced evidence to support the argument that many T6SSs do play a direct role in interbacterial competition.

As mentioned, T6S effectors act on bacterial structures that are both essential and highly conserved. Therefore, these proteins seem to have evolved to exert deleterious effects on a broad array of competitors. The mechanisms by which these proteins attack and disrupt essential pathways further supports their role in antagonism. The structures of amidase-type T6S effectors have revealed open active sites, devoid of inhibitory regulatory domains present in similar housekeeping enzymes[20,21,27–29,51]. The measured activities of effectors support the predictions that these proteins lack stringent regulation, as many studied thus far display activity *in vitro* in the absence of co-activators[15,18,19,27]. Mirroring their *in vitro* activity, T6S-dependent intoxication of cells by amidase and phospholipase effector proteins results in rapid lysis due to cell wall and membrane damage, respectively[27,30,35]. Overall, biochemical analyses of T6S effectors have provided evidence consistent with these proteins participating in antibacterial antagonism.

Genomic and evolutionary evidence further supports the involvement of T6S in interbacterial antagonism. First, immunity proteins are maintained in organisms lacking cognate effector proteins[19]. While these orphan immunity proteins have no demonstrated functionality, their persistence suggests a selective pressure deriving from attack by other organisms. Second, active effector proteins are, without exception, found in the presence of immunity proteins. This underscores the fitness cost associated with effector intoxication in the absence of immunity. Lastly, the role of T6S in interbacterial antagonism is supported by the fact that bacteria-targeting T6S has not been demonstrated in organisms living in privileged sites protected from competition from other bacteria. Moreover, in one example, *Burkholderia mallei*, interbacterial T6SS was lost concomitantly with its evolution from a free-living saprophyte to an obligate pathogen[13].

Observations regarding bacterial antagonism have led Cornforth and Foster to postulate that bacterially encoded antibacterial toxins could be preferentially produced in response to competition-specific stresses[52]. Interestingly, it has been demonstrated both in intra- and inter-species interactions that the activation of the T6SS in one cell can stimulate the system in neighboring cells[30,53]. This recognition is sufficiently integral to the activity of the *P. aeruginosa* H1-T6SS that inactivation of the T6SS of a competitor organism grants resistance to intoxication. Therefore, the intercellular positive regulatory behavior of some T6SSs appears to conform to the predictions of an antagonistic pathway, although it should be noted that not all T6SSs appear to utilize this form of regulation[53].

The conditions that regulate the production of a pathway can provide valuable insights into its physiological function. Some T6SSs, such as the H1-T6SS of *P. aeruginosa* and the *V. cholerae* O1 T6SS, are repressed by quorum signaling[54,55]. This indicates that these systems are active when cells have not established a dense community, and therefore, if operating antagonistically, their function might be to aid in the colonization of surfaces via the displacement of competing bacteria (Figure 2, interaction A). Conversely, other T6SSs, such as the H2-T6SS of *P. aeruginosa*, are induced under conditions of high cell density[54]. These T6SSs could be involved in the defense of communities from invading organisms, or, alternatively, in the invasion of communities of organisms that produce compatible quorum signals (Figure 2, interactions A and B). Additionally, some T6SSs are regulated by environmental cues, such as temperature, pH, or iron availability[56–60]. With respect to

interbacterial antagonism, these signals may indicate the passage of organisms to an environment in which competitor bacteria are present.

Implications of intraspecies antagonism mediated by the T6SS

The more that niche requirements overlap between two organisms, the more probable it is that they will compete in the environment. Therefore, organisms related at the species level are likely to be in fierce competition to occupy mutually favorable niches[61]. While strains sometimes cooperate and form mixed communities, in many cases growth of one strain precludes growth of another, both in the laboratory and in the environment[62–65]. Indeed, antagonism between strains has been studied in depth, and various mechanisms reinforcing competition have been identified[66–68]. Some of these, such as contact-dependent growth inhibition (CDI), closely resemble T6S in that they require close association with target cells and utilize polymorphic toxin–immunity pairs (Box 2)[69].

T6SS-dependent interstrain competition has been directly observed between natural isolates of *S. marcescens* and *V. cholerae*[46,70]. Additionally, some observed E-I pairs are variably present between different strains of a species, such as $Tle5^{PA}$-$Tli5^{PA}$ in *P. aeruginosa,* suggesting that intraspecies competition might be widespread[35,36]. A striking example of the contribution of T6S to interstrain competition is found in the cooperative swarming behavior of *Proteus mirabilis.* Boundaries of dead and dying cells are typically observed when the swarm fronts of non-isogenic *P. mirabilis* strains encounter each other. This phenomenon involves a self-recognition activity that has been linked to three genetic loci, *ids, idr,* and *tss*[65,71]. The *tss* gene cluster encodes a T6S apparatus, and within the *ids* and *idr* loci are genes encoding VgrG proteins and VasX- and Rhs-like proteins, respectively. Consistent with a T6S-like mechanism underlying self recognition in *P. mirabilis*, the process depends on the activity of the *tss*-encoded T6SS and self recognition activity correlates with variability in the *ids* and *idr* loci. Therefore, recognition facilitated by the T6SS appears to directly contribute to the capacity of *P. mirabilis* strains to cooperate.

Interstrain competition might explain the diverse repertoire of T6S E–I pairs in many organisms. Selection to maintain diversity could be due to an arms race, wherein a cell lacking an E–I pair present in its neighbor is rapidly displaced. This further provides an explanation for why a given organism would translocate closely related effector proteins – while these are likely redundant in enzymatic function, they can differ in immune recognition and would be non-redundant with respect to interstrain competition[16,19]. Such processes can ultimately drive speciation. Whereas within contacting populations the acquisition of an E–I pair might lead to the sweeping success of that genotype, kin that contact one-another less often might acquire E–I loci that render them at a standoff. This would affect genetic exchange between organisms, not only by limiting their spatial contact, but also by preventing contact-dependent mechanisms of gene exchange.

Potential roles for T6S beyond antagonism

While significant evidence exists for an antagonistic role for many T6SSs, there remains the possibility that some of these systems might serve purposes beyond competition. One of the most compelling pieces of evidence that indicates additional roles for the T6SS is that it is

often activated under conditions of high cell density[54,55,72,73]. Since clonal expansion can lead to spatial segregation of cells as clusters of closely related groups, these T6S-activated cells are likely to be primarily contacting immediate progeny[74]. Under such conditions, T6S could be utilized for defending the assemblage; however, the significant amount of kin-targeting that must also occur argues that additional hypotheses should be entertained. It is worth noting the diffusible antibiotics are also generally produced under high quorum[52]. Nevertheless, unlike the T6SS these products can act on competitors located at a distance and thus are not restricted to acting on their likely isogenic neighbors.

An instructive frame of reference for generating hypotheses concerning isogenic intoxication is toxin-antitoxin (TA) systems[75,76] and indeed, analogies have been made between TA systems and T6SS effectors[41]. While TA systems are not generally thought to act *in trans* nor depend on additional machinery to exert their effects, when considering only isogenic intoxication there exists the possibility of considerable functional overlap between TA systems and antibacterial T6SS E–I pairs. In this respect it is important to note that TA systems, once considered mainly in the context of plasmid addiction and selfish genetic elements, have recently received much attention for their roles in metabolic regulation, stress responses, biofilm formation, and phage defense[77–82]. It is in light of these, and other findings, that we below examine alternative roles for the T6SS.

Signaling

Many bacteria appear to only transiently exist as independent cells, and instead form complex multicellular communities punctuated by dispersal events[83]. Communication is essential to the establishment and maintenance of these communities; however, previous studies have largely focused on this communication via soluble quorum signaling molecules, and not on contact-dependent mechanisms[84,85]. The latter has the advantage in tight quarters of also providing information concerning the number and the identities of immediately adjacent cells. Notably, in “true” multicellular organisms, direct cell-to-cell signaling is found alongside the production of soluble signaling molecules[86], underscoring the importance of both contact-dependent and contact-independent mechanisms.

It is possible that T6SS effector proteins might play a role in signaling between isogenic cells (Figure 2, interaction C). This could be due to either residual activity of the effector or the E–I complex itself might act as a signaling molecule. The use of toxic effector proteins as signaling molecules is appealing in this respect, as intended recipient organisms successfully interpret the signal, whereas non-intended recipients experience antagonistic effects. Also, the activities of cell wall remodeling effector proteins are consistent with known mechanisms of interbacterial signaling. For example, in *Mycobacterium tuberculosis* and *Micrococcus luteus* cell wall remodeling enzymes have been observed to trigger changes leading to recovery from the viable but non-culturable (VBNC) growth state[87–90]. While Gram-positive organisms can be resuscitated by neighboring cells producing these enzymes, the Gram-negative outer membrane inhibits this mechanism. However, T6SS-dependent delivery of resuscitation enzymes to the periplasm could overcome this and allow active cells to “wake” their neighbors.

Enforcement of social behaviors

Cooperative activities are susceptible to the evolution of non-cooperating organisms, which take advantage of public goods without contributing to the metabolic cost of their production[91,92]. One mechanism that has been proposed to overcome this tendency is enforcement by imposing a cost to non-cooperation. Intoxication by T6SS effectors could be one means by which enforcement is accomplished (Figure 2, interaction D). The known co-regulation of effectors and immunity proteins with social activities would prevent a target organism from failing to cooperate as it would also fail to produce immunity determinants. In *P. aeruginosa* and *P. protegens* it has been observed that E–I loci are within the Gac-Rsm regulon, which also includes a number of social behaviors such as the production of exopolysaccharides[14,32,93–95].

Community structure

Intoxication by effector proteins, while deleterious, might also contribute to the three-dimensional architecture of bacterial communities (Figure 2, interaction E). Indeed, a gross defect in biofilm formation has been observed for T6SS mutants in several organisms[96–98]. In isogenic aggregates, cells undergo differentiation. Some of this differentiation, such as senescence or death with subsequent lysis, mirrors the results of attack by antagonistic molecules[99]. The Tse2 effector of *P. aeruginosa* is bacteriostatic to other *P. aeruginosa* cells and could induce senescence[41]. Interestingly, a role for Tse2 in interspecies competition has not been found, and, as it is present in all sequenced *P. aeruginosa* strains, Tse2 is unlikely to be a mediator of intrastrain competition[14]. Another cell fate, lysis, which releases DNA, an important structural component of extracellular matrices, could likewise be induced by cell wall-degrading or phospholipase effector proteins[15,27,35,100]. Since all cells in an isogenic population would be expected to possess both immunity and effector genes, a mechanism for differential susceptibilities to exchanged effectors must be invoked. Heterogeneity in gene expression is frequently observed in cellular aggregates due to microenvironmental differences found within complex communities[101]. In this manner positional cues might induce differential immunity expression, allowing T6SS-dependent intoxication.

Phage defense

It is known that TA systems can be used to induce suicide in phage-infected bacteria, which protects nearby cells from infection[81]. The importance of this defense mechanism is underscored by the evolution of phage proteins that inhibit the pathway[102]. T6S effector proteins could likewise be used to remove adjacent infected organisms (Figure 2, interaction F). Analogous to TA-mediated phage defense, an infected organism whose cellular machinery has become hijacked to produce phage particles might then become susceptible to effector intoxication as its pre-existing immunity proteins become depleted. The use of T6S for this purpose may be particularly advantageous, as T6S-assisted suicide does not depend on machinery within the infected cell. Thus, this strategy is possibly less susceptible to inhibition by the phage.

Relevant settings for T6S in nature

The studies described in the preceding sections provide intriguing insights into the potential roles of T6SSs in bacterial communities. It will be of interest in future *in vivo* or *in situ* investigations to confirm or dispute predictions based on *in vitro* studies. Below we identify environmental habitats and *in vivo* systems where T6S-dependent interactions are likely to occur. These offer a starting point for probing the role of T6S in natural bacterial communities and for exploring the consequences of manipulating T6-mediated interactions.

T6S in polymicrobial infection

Many organisms with T6S are human pathogens. Initially, T6S was thought to be involved in virulence through direct targeting of eukaryotic cells, but more recent work indicates that targeting eukaryotic cells is a rare property of T6SSs (reviewed in [10]). Accordingly, the role of T6S in virulence is more likely to be an indirect one, stemming from its role in interbacterial interactions. One way in which T6S could contribute to virulence would be by enabling pathogens to compete more effectively with other host-associated bacteria (Figure 3). Precedence for the importance of interbacterial antagonism in bacterial pathogenesis comes from the study of bacteriocins. For example, mice colonized by the bacteriocin-producing probiotic bacterium *Lactobacillus salivarius* are protected from infection by *Listeria monocytogenes*[103]. Bacteriocin-mediated interactions were also found to predict the outcome of competition between strains of *Streptococcus pneumonia* in the mouse nasal pharynx[104].

Antagonistic interactions mediated by the T6SS could be important for bacterial pathogenesis in a variety of contexts (Figure 3). First, in order to establish an infection, pathogens must be able to overcome the colonization barrier presented by the native microflora. This is especially important for enteric pathogens competing against established populations in the intestine[105]. Although T6S is not known to target Gram-positive organisms, which constitute much of the intestinal microbiota, the numerous enteric pathogens with T6SSs, including *Salmonella enterica*, *C. rodentium, Aeromonas hydrophila,* and enteroaggregative *Escherichia coli* nonetheless suggests an adaptive role for the system in the gut[48,106–109]. One role may be to selectively target the proteobacterial commensals, such as beneficial *E. coli* strains, that occupy mutually favorable niches. The system may additionally target Bacteroidetes, a highly abundant phylum in the gut; however, its activity against non-proteobacterial Gram-negatives is yet to be determined.

When the commensal colonization barrier is compromised, for example by the disruption of an epithelial surface, the host can become susceptible to infection by many bacteria. In this context, T6S could give pathogens a competitive advantage over other potential colonizers. For example, organisms with bacterial-targeting T6SSs, including *A. baumanii*, *P. aeruginosa, S. marcescens*, and *Proteus mirabilis*, are common inhabitants of wounds[14,46,50,71,110–112]. Following stable colonization, it is conceivable that T6S further enables pathogens to defend their niche by resisting invasion by incoming competitors, of the same or other species (Figure 3). In the lungs of cystic fibrosis (CF) patients, for example, *P. aeruginosa* infections can persist for years, overall diversity of the species colonizing this habitat appears to decrease as patients age and lung damage accumulates,

and populations of *P. aeruginosa* in the CF lung are typically clonal[113–116]. Accordingly, T6S is one mechanism by which *P. aeruginosa* populations could be preventing invasion by other organisms. Consistent with a role for T6SS in CF infections, clinical isolates of *P. aeruginosa* from CF infections frequently have highly active antibacterial T6SSs, and serum from CF patients has been shown to react to Hcp1[95,117].

Chronic infections, such as those in CF lungs or diabetic wounds, also represent a habitat that may support functions of T6S beyond bacterial antagonism. Bacterial populations in chronic infections often exist within structured aggregates[118,119]. As described above, the microhabitats within such structures could lead to differential gene expression that facilitates T6-based signaling or cellular differentiation.

T6S in environmental populations

Many organisms with T6SSs are not human pathogens. Additionally, the human pathogens with T6SSs are not typically specialists; rather, they are opportunistic pathogens that also inhabit environmental niches outside of the hosts they infect [10]. Therefore, T6S likely plays an important role in bacterial competition outside of disease settings. Given that T6S requires intimate contact between cells, it is most likely to be important in habitats where bacteria predominately form microcolonies or aggregates. The phyllosphere and the rhizosphere both represent habitats colonized by diverse bacterial species that are often found clustered together[120,121]. The plant pathogen and leaf surface colonizer *P. syringae* DC3000 tomato has two T6SSs, one of which has been shown to be important for bacterial antagonism *in vitro*[49,122]. T6SS gene clusters are also common among species from the plant-associated genera *Erwinia*, *Pantoea,* and *Pectobacterium*, and *hcp* and *vrgG* genes in *P. atrosepticum* are induced by potato stem extract[123,124]. Intriguingly, one study suggests that T6S could be important for bacterial interactions in bulk soil not associated with plant roots. A T6SS mutant of *P. fluorescens* Pf0–1 grew less well than the wild-type in soil containing indigenous bacteria, but achieved similar growth yields to wild-type in sterilized soil[125].

Conclusions

Many of the recent advances in our understanding of the function of T6S have come about through the identification and characterization of its secreted effectors. The vast majority of effectors identified to date exhibit antibacterial activity toward susceptible recipients, underscoring the importance of T6S in mediating interbacterial interactions. The broad range of the targets of T6S effectors further strengthens the argument for the general importance of the antagonistic function of T6SSs. However, a more nuanced examination of the potential physiological roles for T6S indicates that they extend beyond antagonism to include other effects on bacterial populations.

The potential for T6S to influence microbial community composition suggests tantalizing avenues for its application. The need for new antimicrobials, particularly those with the ability to target chronic, recalcitrant infections, has never been more apparent. The ability of T6SSs to deliver potent antimicrobials directly to Gram-negative pathogens makes the system an attractive candidate for the engineering of novel antimicrobial mechanisms into

probiotic organisms. Such an antibacterial approach would benefit from the capacity of T6S to act in a biofilm, a growth state notoriously difficult to treat due to its enhanced resistance to traditional antimicrobials [13,126]. Depending on the contribution of a given T6SS to the *in vivo* fitness of a bacterial pathogen, an alternative strategy could be to develop inhibitors of its activity. These inhibitors would combat infection by decreasing the ability of pathogens to compete against resident microflora. Finally, the ability of T6S to contribute to bacterial fitness could be harnessed by augmenting the effector repertoires of environmentally beneficial organisms, such as plant growth promoting bacteria or bioremediation species, to facilitate their ability to compete with indigenous organisms.

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Glossary

Proteobacteria	Diverse phylum of Gram-negative bacteria containing most identified T6SS-positive organisms. The T6SS is found distributed throughout all five classes of this phylum: α, β, γ, δ, and ε
Periplasm	A Gram-negative bacterial compartment found between the outer and inner membranes that contains the cell wall
Type IV secretion system	Gram-negative secretion system involved in the transfer of both DNA and proteins to bacterial and eukaryotic targets
Hcp	Ring-shaped substrate and structural component of the T6SS. Structurally related to the T4 bacteriophage tail tube protein gp19
Effector	A translocated bacterial protein that mediates a specific effect on the recipient organism
Peptidoglycan	Major structural component of bacterial cell walls, consisting of glycan strands of alternating β1-4-linked N-acetylglucosamine and N-acetylmuramic acid that are connected by peptide crosslinks
Sacculus	Term for the total cell wall structure (peptidoglycan and associated molecules) of a Gram-negative bacterium, derives from the appearance of isolated cell wall superstructures as meshwork bags
VgrG	Valine-glycine repeat protein G. Substrate and structural component of the T6SS. Structurally related to the T4 bacteriophage tail spike apparatus, gp27 and gp5
Bacteriocin	Proteins released by bacteria that exert toxic effects on related organisms – are found encoded adjacent to specific immunity determinants that protect the producing cell

Rhs	Large multi-domain proteins with a central element consisting of a repeating motif – initially identified as sites of frequent recombination within the *E. coli* genome. Often appear to bear a toxic C-terminus
Quorum signaling	An interbacterial signaling system in which signal concentration correlates with cellular density, thus producing a measure of the local concentration of signal-producing organisms
TA systems	System consisting of a toxic element and an unstable cognate antitoxin that mediates toxicity within the producing cell such that depletion of the antitoxin results in death or senescence of the organism
VBNC	State in which a cell is not actively dividing but maintains viability
Cooperation	Investment of resources by one individual into a process that benefits other individuals. Cooperation is not necessarily mutually beneficial to both partners
Cystic fibrosis	Recessive genetic disorder arising from mutations affecting function of the CFTR (cystic fibrosis transmembrane conductance regulator) protein. Hallmarks of cystic fibrosis include dehydrated secretions at a number of mucous membranes, poor growth, and chronic lung infections
Phyllosphere	Habitat consisting of the above ground surfaces of plants, particularly the leaves
Rhizosphere	Habitat consisting of plant roots and the surrounding soil influenced by their secretions

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Box 3

T6S and CDI; analogous, but not functionally redundant

Although versatile, the T6SS does not typically provide bacteria the full complement of contact-dependent competitive and cooperative mechanisms. Gram-negative bacteria therefore encode additional pathways that may in certain instances share properties with T6S, yet these pathways possess distinct, specialized capabilities. One of these, contact dependent inhibition (CDI), uses a two-partner secretion mechanism to present filamentous toxins, termed CdiA proteins, at the cell surface (reviewed in Ruhe et al[69]). According to the prevailing model, this positions the C-terminal toxin domain distal to the donor cell such that it is poised to engage and enter the target cell. Like T6S effectors, the toxin domain of CdiA is highly polymorphic – even within families that exhibit the same enzymatic activity[139–141]. Also similar to the T6SS, the CDI system self-targets; therefore, equally polymorphic cognate immunity proteins protect against self-intoxication.

CDI systems are common in Proteobacteria, where they are often found in co-occurrence with T6S. Why then might an organism possess these seemingly functionally redundant pathways? Whereas the T6SS targets Gram-negative bacteria in a largely indiscriminate fashion, CDI is so far known only to occur between highly related bacteria – likely due to its requirement for outer membrane receptors that vary between bacterial species[142]. Therefore, CDI does not serve directly as a broad defense mechanism and instead appears more restricted in function. Data also suggest that the physical and temporal constraints on CDI function might be more permissive than those of T6S, allowing CDI to function under circumstances not favorable to T6S. For instance, CDI-dependent fitness differences can be observed between bacterial populations cultivated in liquid medium, while even constitutive, highly active T6S effector donor strains appear to display no capacity to target sensitive recipients under these conditions[14,143]. A possible explanation for this difference is that transient contacts brought about by cellular collisions do not provide the time needed for the assembly of an appropriately oriented, complex secretory apparatus like the T6SS. It is currently difficult to compare the kinetics of intoxication by these two systems directly. However, the toxin domain of CdiA is readily observed in the cytoplasm of target cells within one hour co-cultivation with donor cells, while under similar conditions the lysis of recipient cells by the T6SS does not occur robustly until well after one hour[30,144]. As we further unravel the intricacies of these two pathways, it is likely additional insights into their divergent functions and evolutionary significance will be made.

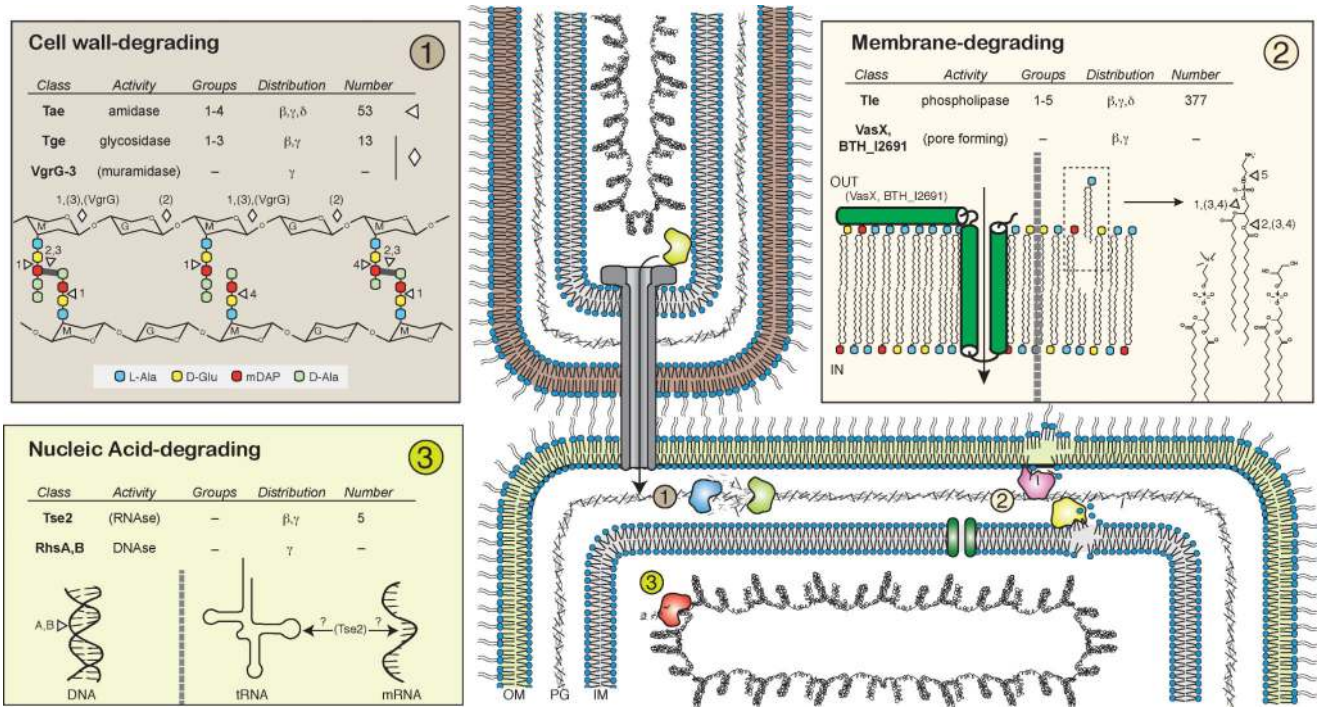

Figure 1. T6SS effectors target varying aspects of bacterial physiology

Localization and activity of interbacterial T6S effectors delivered by an attacking donor cell (red) to a recipient cell (green) via the T6S apparatus (grey tube). Effector targets include nucleic acids, the peptidoglycan cell wall (PG), the inner-membrane (IM), and the outer-membrane (OM). Precise enzymatic specificity, where known, is further indicated within numbered panels. Parentheses indicate enzymatic activities predicted from structure-function and/or nonspecific enzymatic analyses that have yet to be biochemically confirmed. Distribution denotes effector presence within the different proteobacterial classes (, , , , or). Group refers to the number of evolutionarily distinct families of effector proteins within an enzymatic class, and number to the unique instances of homologs within those groups. Numbers presented are limited to those reported in the literature; groups and numbers are provided only when a systematic effort has been made to characterize a class of effectors[14–16,18,19,32,35,41,43,134]. Dashes indicate a lack of available published data. Abbreviations: M, N-acetylmuramic acid, G, N-acetylglucosamine, mDAP, *meso*-diaminopimelic acid.

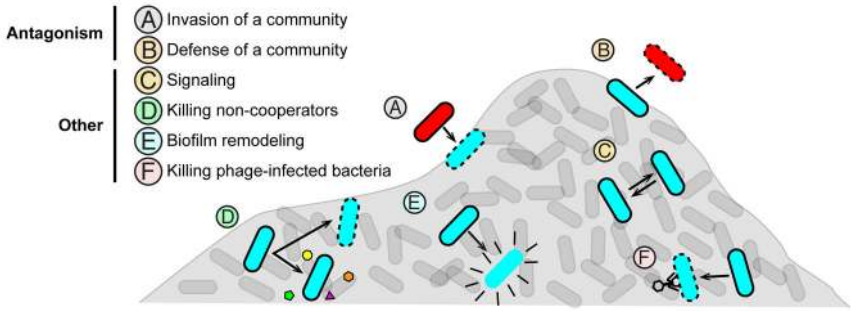

Figure 2. Multiple roles for interbacterial T6S

Potential activities of T6S within an established bacterial community, both antagonistic (between red and blue competitor cells) and non-antagonistic (between two blue cells). Dashed outlines indicate cells experiencing T6-mediated toxicity, a lack of an outline indicates a cell lysing due to the activity of T6SS effectors. Arrows represent the directionality of T6S interactions.

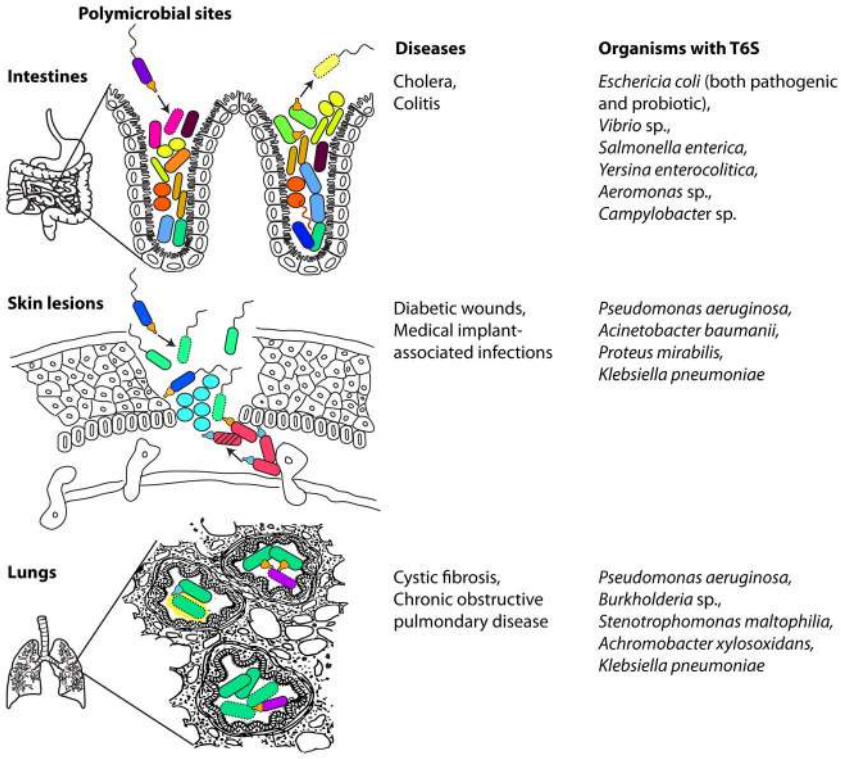

Figure 3. Interbacterial T6S and infection

Potential roles for T6S-mediated interactions in disease. Both antagonistic and alternative functions for T6S are depicted. The T6SS appears as an additional structure on cells; it is orange when mediating an antagonistic function, and blue when its activity has an alternative function. Cells being attacked by the T6SS of others are depicted with a dashed outline. Stripes across a cell indicate T6S-mediated change in physiology, and a yellow halo around a cell depicts intracellular contents released upon lysis. In the intestine, T6S might be used by invading pathogens (intestine close-up, left) or by commensals blocking invasion (intestine close-up, right). In skin wounds T6S could be important for competition during colonization (wound closeup, blue vs. green cells), could allow established populations to protect their niche from susceptible invaders (wound close-up, red vs. green cells), or could facilitate signaling within populations (red cell targeting another red cell). In chronic lung infections, roles for T6S could include preventing invasion of an established population by susceptible species (lung cross-section, upper right), facilitating invasion of a susceptible established population (lung cross-section, bottom), or contributing to aggregate structure by mediating the lysis of a subpopulation of cells (lung cross section, upper left).