REVIEW
Unni *et al.*, *Microbiology* 2022;168:001151
DOI 10.1099/mic.0.001151

# Presence and absence of type VI secretion systems in bacteria

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**Abstract**

The type VI secretion system (T6SS) is a molecular puncturing device that enables Gram-negative bacteria to kill competitors, manipulate host cells and take up nutrients. Who would want to miss such superpowers? Indeed, many studies show how widespread the secretion apparatus is among microbes. However, it is becoming evident that, on multiple taxonomic levels, from phyla to species and strains, some bacteria lack a T6SS. Here, we review who does and does not have a type VI secretion apparatus and speculate on the dynamic process of gaining and losing the secretion system to better understand its spread and distribution across the microbial world.

## INTRODUCTION

The type VI secretion system (T6SS) is a remarkable molecular machinery for the translocation of proteins into neighbouring cells or the extracellular space (Box 1, Fig. 1) [1]. The secreted proteins (called effectors) have diverse functions, resulting in the killing or inhibition of growth of prokaryotic and fungal cells, the manipulation of eukaryotic cells, or the uptake of nutrients [2–7]. While the number of bacteria known to possess a characterized T6SS is rapidly increasing, it is also becoming evident that not all bacteria have such a secretion apparatus. Even among strains of the same species, some do and others do not have a T6SS. 'Who has a T6SS?' is therefore not a trivial question, and one of utmost importance, considering the role of the secretion apparatus in bacterial ecology [8] and pathogenicity [9].

The constantly growing number of sequenced bacterial genomes and the development of customized bioinformatics tools have enabled large-scale analyses of T6SS genes across bacteria [10–14]. Ready-to-use applications allow the screening of genomes for the presence of T6SS genes [15]. Comparative genomics has revealed homologies between different T6SS gene sequences and identified genes unique to particular T6SS subtypes (Box 1) [16].

Bacterial sampling and isolation from natural habitats have led to a better understanding of T6SSs beyond laboratory reference strains. Sampling from multiple geographical sites and ecosystems has revealed environments in which bacterial strains with the T6SS are more common than strains of the same species without the secretion system [17, 18]. Collection of isolates from natural habitats across time enabled the analysis of changes in the T6SS, and in its prevalence and distribution over time [19–21].

Experiments in the laboratory resulted in a better molecular understanding of the secretion system. Advanced microscopy led to the discovery of an additional T6SS subtype and revealed its structure at high resolutions [13]. Experiments with T6SS-carrying plasmids provided proof of principle for the gain of the T6SS through horizontal gene transfer (HGT) [17, 22]. Functional assays revealed how mutations in T6SS-encoding genes affect the function or activity of the secretion system [19, 21].

A growing body of theoretical work and mathematical models is addressing why bacteria are better off with or without a T6SS. Modelling studies have defined parameter spaces in which T6SS-mediated activities are beneficial [23–27], while theoretical studies have provided a framework for competition between bacteria and the costs and benefits associated with bacteria–bacteria killing [28–31].

Here, we aim to review the latest literature on the prevalence of T6SSs and to explain their diverse presence/absence patterns by considering (i) the molecular mechanisms underlying the spread of T6SSs and (ii) the factors that interfere with T6SS acquisition or drive T6SS loss. We exclusively focus on genes encoding the T6SS apparatus (Box 1) and refer to existing reviews for variations in T6SS effectors [32–37]

Received 09 December 2021; Accepted 01 February 2022; Published 25 April 2022
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**Keywords:** type VI secretion system; comparative genomics; intraspecific variation; horizontal gene transfer; mobile genetic elements; presence/absence variation; T6SS subtypes.
**Abbreviations:** FPI, *Francisella* pathogenicity island; HGT, horizontal gene transfer; ICE, integrative and conjugative element; SPI, *Salmonella* pathogenicity island; T3SS, type III secretion system; T6SS, type VI secretion system.

or variations as a result of differential regulation [38–44]. We consider a bacterium 'T6SS-positive' if it carries the T6SS apparatus genes and as 'T6SS-negative' if it does not carry these genes. Before analysing T6SS-positive bacteria taxonomically, we take a bird's-eye view of the T6SS and what is known about the secretion system across ecosystems.

## T6SSs ARE PREVALENT IN BACTERIA ACROSS ECOSYSTEMS AND CLINICAL SETTINGS

The T6SS is prevalent across bacteria in particle-attached communities and entirely free-living bacteria in marine and soil ecosystems (Fig. 2). Kempnich *et al.* detected multiple genera of T6SS-positive bacteria in samples from the Pacific Ocean [20]. *Vibrio corralliilyticus, V. anguillarum* and *V. alginolyticus* are just a few examples of aquatic *Vibrio* species that encode T6SS genes and use the apparatus to kill bacterial competitors, as shown in laboratory experiments [45–47]. In the soil, the bacterium *Pseudomonas putida* carries T6SS genes and is known to kill a broad range of bacteria in a T6SS-dependent manner, often in favour of the plants with which *P. putida* are associated [48]. Another soil bacterium, *Myxococcus xanthus*, uses its T6SS to attack sibling cells under starvation, thereby maintaining a population of healthy, well-fed cells [49]. While there are free-living bacteria with the T6SS, comparative studies suggest that the T6SS is less common among free-living bacteria and more prominent among complex microbial communities, with even higher prominence among host-associated microbial communities [14, 20].

Some T6SS-positive bacteria are associated with eukaryotic hosts as members of the microbiomes of plants and animals (Fig. 2). In plants, symbiotic and plant growth-promoting bacteria with T6SS genes have been described [50, 51]. *Rhizobium leguminosarum* is an example for a symbiotic plant-associated bacterium that requires its T6SS for plant colonization, probably by killing other plant root-associated microbes, and for efficient nitrogen fixation [52]. *Pseudomonas protegens* is a plant-protecting bacterium with a T6SS. It uses the secretion

**Box 1. The type VI secretion system**

The T6SS is a dynamic multiprotein complex formed of more than 10 proteins, the exact number depending on the particular T6SS subtype (Fig. 1a, b). 'T6SS' in this review broadly refers to the secretion apparatus formed by these proteins. At its core, the secretion system consists of an inner tube (of Hcp, also referred to as TssD) surrounded by a contractile outer sheath (formed of TssB and TssC, also referred to as VipA and VipB) [99, 131, 133]. The sheath is rooted in a baseplate (of TssE, F, G, K) and may span the entire cytoplasm of the bacterium [134, 135]. TssJ, L and M comprise the transmembrane complex. Effector proteins are situated at the tip (formed of VgrG and PAAR domain proteins) or inside the inner tube [136]. Contraction of the outer sheath results in the ejection of the inner tube (and its associated effectors) into the extracellular space or directly into neighbouring cells in the vicinity (Fig. 1c, d). After contraction, the ATPase TssH (also known as ClpV) dissociates the outer sheath and prepares its components for reassembly [105].

Four subtypes of the T6SS exist, namely T6SS$^{i}$ [10], T6SS$^{ii}$ [87], T6SS$^{iii}$ [11] and T6SS$^{iv}$ [13]. While their coding sequences differ, all four show structural and functional similarities, with certain features being unique to individual subtypes (Fig. 1e) (reviewed in more detail in [8]). T6SS$^{i}$ stands out among the other subtypes with TssA proteins that anchor the rear end of the secretion system in the cell membrane, allowing for sheath formation and time delay until firing [16, 137 138, 137]. T6SS$^{ii}$ differs from the other subtypes by the lack of the base plate component TssE [11]. T6SS$^{iii}$ is characterized by the lack of homologues to known proteins that form the membrane complex (TssJ, L, M) and encodes other proteins that might do so instead (TssN, TssO, TssP) [11]. T6SS$^{iv}$ differs in multiple aspects from the other subtypes, and more closely resembles existing extracellular contractile injection systems [13]. This subtype lacks the membrane complex of T6SS$^{i}$ and T6SS$^{ii}$, encodes a putative tail terminator (A72) and tape measure protein (A06), and builds arrays of tube and sheath proteins that are aligned next to each other to fire in the same direction [13]. TssH is also missing in subtype IV [13]. As described in the main text and) (Fig. 3a), a striking difference between all four subtypes other than their assemblies is their prevalence among bacterial taxa.

A given bacterium can harbour multiple T6SSs. Often, the different T6SSs are phylogenetically distinct and were probably acquired independently, are regulated differently, and are not functionally redundant [10, 139].

By translocating effectors into other cells or into the extracellular space, the T6SS enables bacteria to interact with their biotic and abiotic environment. The impact of the secretion system depends entirely on the secreted effector proteins and their enzymatic activities. A multitude of effectors with a wide variety of activities have been reported [32]. Once secreted into prokaryotic or fungal cells, effectors degrade lipids, peptidoglycans and DNA, or form pores, thus exerting a toxic effect on the target cells [32, 140, 141]. This is how the T6SS mediates killing of bacteria and fungi [2, 3]. When fired into eukaryotic cells, effectors are known to engage in diverse activities that affect intracellular signalling pathways and cell shape [4, 142]. Effectors secreted into the extracellular space have been reported to directly bind metal ions or to facilitate the docking of outer membrane vesicles loaded with metal ions to the cell, and thereby aid in nutrient acquisition [5, 73]. For effectors with anti-prokaryotic activities, immunity proteins are expressed, many of which are encoded next to their cognate effectors, which specifically inhibit the toxic effector activity [2]. This allows bacteria to engage in T6SS-mediated interactions without killing each other, and the bacteria are therefore considered compatible [92].

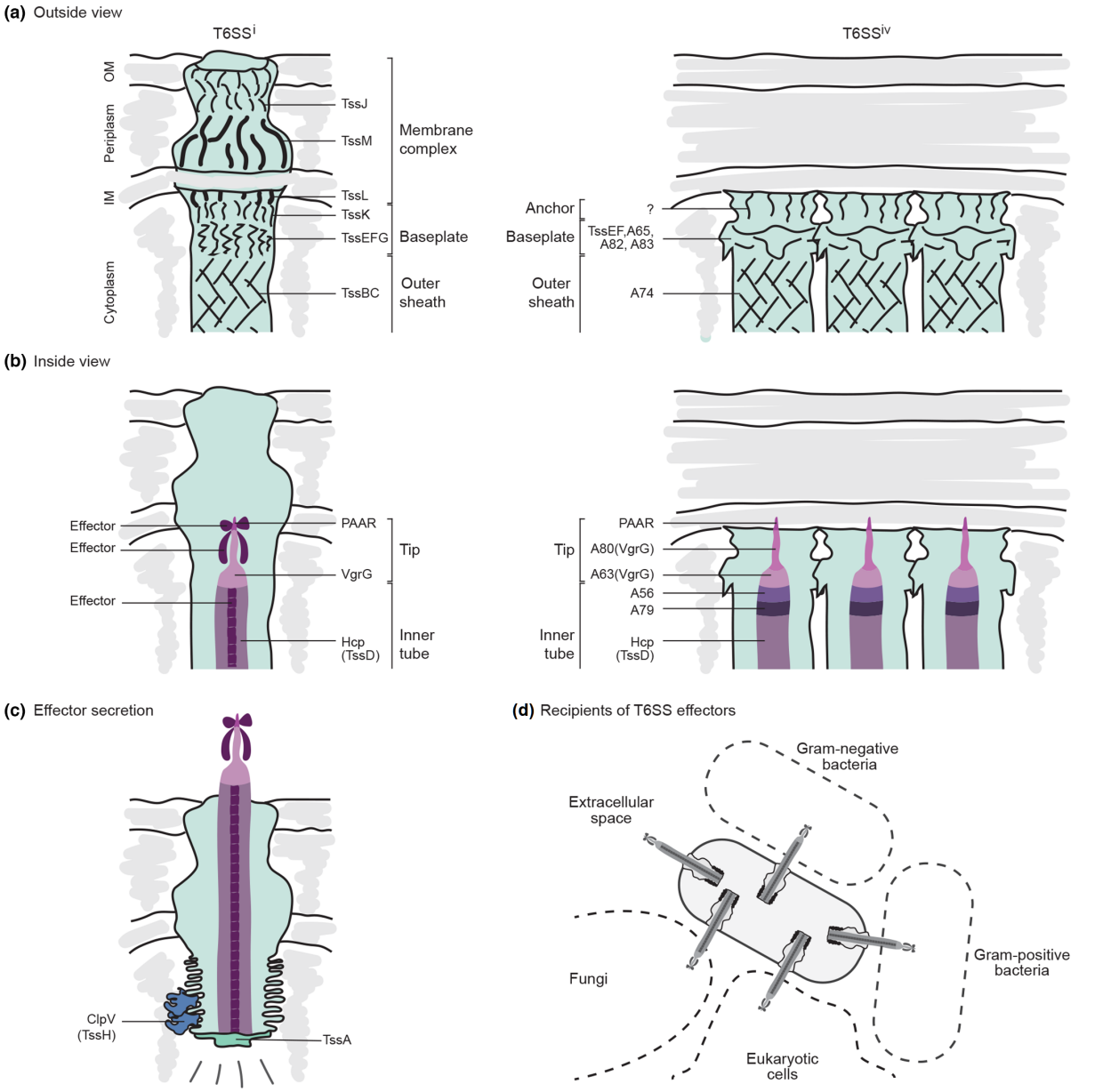


**Fig. 1.** The structure and genetics of the T6SS. (a, b) Cartoon schematic of the structure of the T6SS before secretion. The figure shows the T6SS$^{i}$ and T6SS$^{iv}$ with an extended outer sheath, with the component proteins labelled. The drawings are based on existing structures of T6SS components [13, 143–147]. Artistic freedom was granted. Effectors are expected to be associated with the T6SS$^{iv}$ apparatus similar to that in T6SS$^{i}$ and were not indicated because of the lack of available images. As the *Amoebophilus* T6SS$^{iv}$ genes are not yet named, we have labelled components with abbreviations of the first letter and last two digits of the locus tags in reference to table S4 from Böck *et al.* [13] when homology to known T6SS components was unclear. IM: inner membrane, OM: outer membrane. (c) Contraction of the outer sheath results in ejection of the inner tube and its associated effectors to the outside of the bacterium. Although indicated here for T6SS$^{i}$ only, this mechanism is highly conserved across subtypes. (d) T6SS effectors are either secreted into the supernatant or directly translocated into a variety of cells within reach of the bacterium. (e) Genetic makeup of the four T6SS subtypes. Dashed boxes show genes that are present in only one T6SS subtype, and solid boxes show genes with known homologues in at least two T6SS subtypes. Genes in the same column are homologues. The genetic organization differs from the schematic depiction and varies within subtypes. Figure based on information from the literature [8, 13, 148].

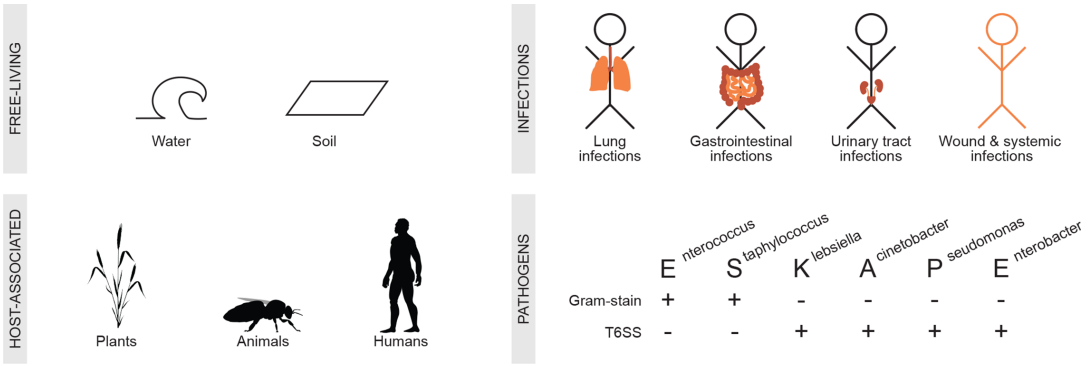


**Fig. 2.** Prevalence of bacteria with a T6SS across ecosystems. Free-living bacteria and members of particle-attached microbial communities in marine and soil ecosystems encode T6SS genes. Host-associated bacteria with a T6SS are represented in the microbiomes of plants and animals, including humans. Pathogenic bacteria that cause a variety of diseases encode T6SS genes. T6SSs are present in all Gram-negative members of the ESKAPE pathogens, which are of concern to human health because of their multidrug resistances. Source of some depictions: http://phylopic.org/.

system to invade and kill insects that feed on the plant by competing with bacteria in the insect gut microbiome [53]. Another plant-associated microbe, *Pseudomonas taiwanensis*, has been shown to use its T6SS to mediate colonization resistance against bacterial plant pathogens through T6SS-mediated secretion of pyoverdine, an iron chelator [54]. Among animals, T6SS-carrying bacteria are represented in the microbiota of squids, bees and humans, among others (Fig. 2) [55–57]. The bobtail squid is an elegant example for symbiosis with the T6SS-positive *Vibrio fischeri*. *V. fischeri* colonizes the squid light organ, rendering the squid less visible to its predators, using its two T6SSs to kill other bacteria and gain dominance [55, 58]. Studies on the three T6SSs of *Bacteroidales* have suggested that T6SS-positive bacteria may play a role in the colonization of the human microbiome [57, 59]. *Bacteroidales* are a highly abundant order of microbes in the human gut microbiome, members of which have been reported to encode the T6SS from human samples worldwide [60]. Thus, T6SS-positive bacteria are highly abundant in host-associated communities, where they use the T6SS, among other things, to kill competing bacteria. Among members of the microbiota, T6SS-mediated killing can contribute to their spatial organization. Alternatively, T6SS-mediated killing can also mediate colonization resistance against pathogens, which may encode T6SS genes themselves.

T6SS-carrying bacteria are also common among pathogens. Human infections associated with T6SS-positive bacteria include lung infections, gastrointestinal infections, urinary tract infections, and wounds and systemic infections (Fig. 2) [1, 61, 62]. The so-called 'ESKAPE' pathogens are of high concern due to their high rate of nosocomial infections and multi-drug resistance [63]. All four Gram-negative ESKAPE species (namely *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* and *Enterobacter cloacae*) have one or several T6SSs (Fig. 2) and are discussed in more detail here. *K. pneumoniae* is an opportunistic pathogen and a resident of mouth, skin and intestinal flora. When aspirated into the lung, it can cause life-threatening infections, especially in immunocompromised individuals [64]. *K. pneumoniae* has been shown to use its T6SS to kill bacterial and fungal competitors. Large-scale *in silico* analyses revealed extensive diversity of T6SS loci in *K. pneumoniae* strains, with some strains having multiple T6SS loci [65]. *A. baumanii* is an opportunistic pathogen that causes a broad range of infections, including of the skin and soft tissue, urinary tract infections, meningitis, and pneumonia [66]. *A. baumanii* also uses its T6SS for interbacterial competition [67]. *In silico* analyses revealed a high diversity of T6SS effectors in *A. baumanii*, the functions of many of which remain unknown [68]. The T6SS of *A. baumanii* has been directly linked to multidrug resistance. Di Venanzio and colleagues showed how T6SS activity can be repressed in *A. baumanii* by a plasmid that encodes antibiotic resistance genes [69, 70]. *P. aeruginosa* is an opportunistic pathogen that causes a wide array of infections, including soft tissue infections, urinary tract infections, bacteraemia and pneumonia [71]. *P. aeruginosa* was one of the first species in which a T6SS was described [61]. The *P. aeruginosa* T6SS is known to be associated with interbacterial competition [2], anti-eukaryotic activity [72] and nutrient acquisition [73, 74]. Proteins of the *P. aeruginosa* T6SS were detected in the sputum of cystic fibrosis patients, indicating that the T6SS was active during lung infections [61]. The *E. cloacae* complex comprises diverse polyphyletic species that are phenotypically too similar to resolve [75]. They are opportunistic pathogens that contribute to many infections, including bacteraemia and soft tissue, respiratory tract, urinary tract and intestinal tract infections [76]. Genomic analyses of several *E. cloacae* strains revealed the presence of at least one T6SS locus in every strain. One *E. cloacae* strain encoded two T6SS loci, and *in vivo* experiments in mice suggest that both T6SS loci contributed to gut colonization and pathogenesis [77]. Besides human pathogens, T6SSs are also present among animal and plant pathogens (as reviewed in [15, 21, 78]). Taken together, the T6SS is prevalent among pathogenic bacteria, and, in some cases, directly contributes to virulence, either by competing against resident microbes during colonization or by directly harming the host. In this section, we have discussed the prevalence of the T6SS across microbes in various ecosystems and lifestyles. It may therefore seem paradoxical to observe that the T6SS genes show presence/absence variation at different taxonomic levels, from phylum to strain. In the following sections, we discuss this presence/absence variation and speculate on factors that may affect it.

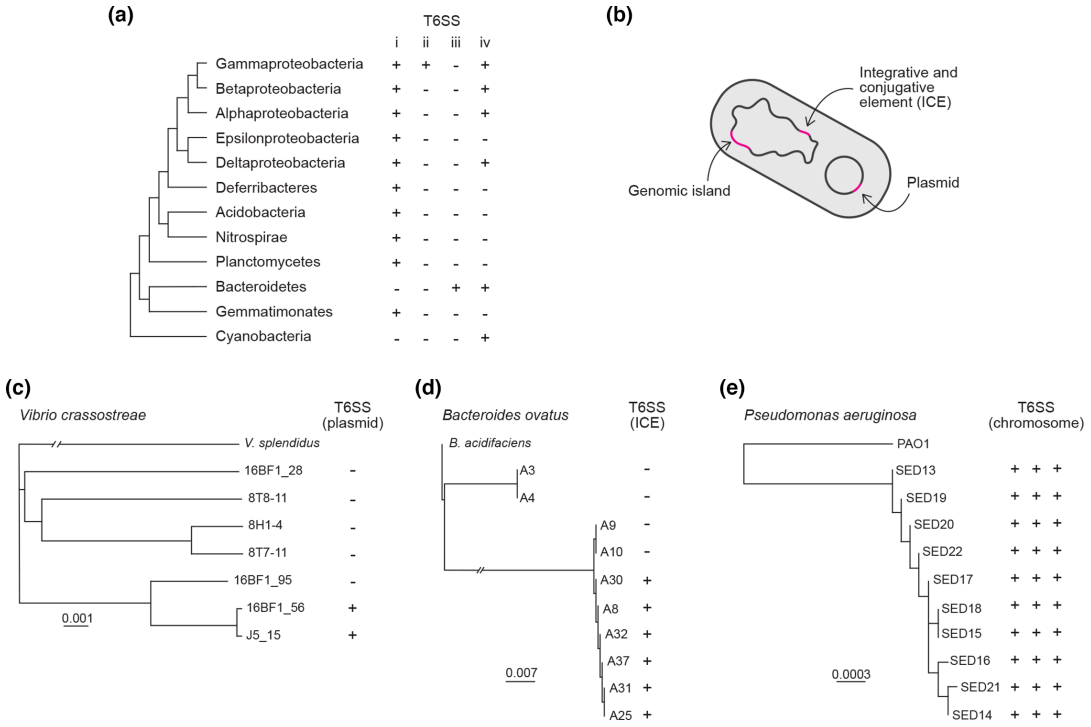


**Fig. 3.** T6SS presence/absence variation at multiple taxonomic levels. (a) Presence/absence of T6SS subtypes across T6SS-positive phyla. Subtypes were assigned based on [11, 13, 148]. Sketch tree is based on [149]. (b) Depiction of genomic elements with T6SS genes. Genes of the secretion system are found on genomic islands, on integrative and conjugative elements (ICEs), and on plasmids. (c) Intraspecific T6SS presence/absence variation in *V. crassostreae* isolated from the marine ecosystem at Brest (France). Some isolates carry the plasmid pGV with T6SS genes, while other isolates do not. Only a subset of the isolates are shown; see [80] for a more comprehensive overview. The phylogenetic tree was built based on whole genome sequences using the program andi [150], and *V. splendidus* was used as an outgroup. (d) Presence and absence of T6SS in *B. ovatus* isolated from the gut microbiome of one individual (referred to as 'af', BIOML cohort). Some isolates carry ICEs with T6SS genes (type GA2c), whereas others do not. See [60] for the entire study on T6SS prevalence in *Bacteroides*. The phylogenetic tree was built based on whole genome sequences using andi [150], and *B. acidifaciens* was used as an outgroup. (e) Phylogenetic tree of *P. aeruginosa* isolates as an example of a species with no reported intraspecific T6SS presence/absence variation. Each isolate encodes three T6SSs on the chromosome, which are called H1, H2 and H3. All isolates are derived from one patient [151]. Whole genome sequences were analysed for T6SS genes via MacSyFinder [15] and were used to build the phylogenetic tree via andi [150]. The reference strain PAO1 was used as an outgroup.

## PRESENCE AND ABSENCE OF T6SSs ACROSS BACTERIAL TAXA

At the phylum level, T6SS genes have been detected in genomes belonging to fewer than ten of the over 100 currently classified phyla (Fig. 3a) [1, 10–14, 79]. The T6SS is therefore a trait present only in a small fraction of phyla. Experimental evidence has been published for T6SS-positive members of the *Proteobacteria* [1] and *Bacteroidetes* [11], while only bioinformatics-derived evidence exists for T6SS in *Acidobacteria*, *Deferribacteres*, *Nitrospirae*, *Planctomycetes*, *Gemmatimonates* and *Cyanobacteria* [11–13]. A very recent analysis in preprint by Geller and colleagues also identified T6SS genes in genomes from *Actinobacteria*, *Verrucomicrobia*, Candidatus Aminicenantes and Candidatus Eisenbacteria, which requires further validation [14]. All validated T6SS-positive phyla described to date have a diderm and are Gram-negative. The phylogenetic distribution of T6SS-positive phyla suggests that they may not all be closely related or even monophyletic.

Within each phylum, only a fraction of bacteria are T6SS-positive [12, 14]. Generally, less than half of the analysed genomes within a given phylum are T6SS-positive [12]. At the genus level, *Francisella* stands out as the only genus among the *Gammaproteobacteria* with the T6SS$^{ii}$ [11] (Fig. 2a). At the strain level, various presence/absence patterns can be observed, too. In *Vibrio crassostreae*, for example, some strains are T6SS-positive and others T6SS-negative (Fig. 3c), as is the case with *Bacteroides ovatus* as well (Fig. 3d) [60, 80]. Indeed, intraspecific presence/absence variation of the T6SS was observed in all *Bacteroides* species analysed by García-Bayona and colleagues [60]. In most of the analysed species, roughly 10–80% of the strains were T6SS-positive. In other species such as *P. aeruginosa* or *V. cholerae*, all strains analysed to date carry T6SS genes (Fig. 3e). Taken together, the T6SS has different distribution patterns at different taxonomic levels, raising the question of how and why some, but not other strains carry T6SS genes. In the following sections, we discuss three notable factors that vary between bacteria with differing presence/absence patterns: T6SS subtype, genomic environment of T6SS genes and location of bacteria.

## VARIATIONS IN T6SS SUBTYPES

Presence/absence patterns of T6SSs at the phylum and genus levels might be explained by their subtypes (Box 1). Different T6SS subtypes show differing prevalence levels across phyla (Fig. 3a). Subtype I (T6SS$^{i}$) is prevalent across multiple bacterial phyla and a number of proteobacterial genera, including *Vibrio* [1], *Pseudomonas* [61], *Burkholderia* [81] and *Acinetobacter* [67]. Some, but not all, species of bacteria with T6SS$^{i}$ show presence/absence variation. T6SS subtype II (T6SS$^{ii}$) has only ever been reported in the genus *Francisella* [12, 82, 83]. As the *Francisella* pathogenicity island (FPI), which encodes T6SS genes, is present in all recorded subspecies of *Francisella*, it is likely that it comprises a core part of the genome. The T6SS subtype III (T6SS$^{iii}$) is restricted to the phylum *Bacteroidetes*, but has been reported in multiple genera, including *Bacteroides*, *Flavobacterium*, *Prevotella* and *Sphingobacterium,* and inter- and intraspecies presence/absence variation have also been reported [12, 60]. The T6SS subtype IV (T6SS$^{iv}$) is prevalent in diverse bacterial phyla [13]. Altogether, it is unlikely that the structural differences between the T6SS subtypes (Box 1) are causal to their different distribution patterns; rather, the subtypes might reflect adaptations to the different bacterial hosts and their respective lifestyles across the bacterial kingdom. Alternatively, different subtypes might be indicative of independent ancestral acquisition events.

## VARIATIONS IN THE GENOMIC ENVIRONMENT IN WHICH T6SS GENES ARE ENCODED

Among T6SS-positive bacteria, there is some variation in the genomic environment (such as plasmids, mobile genetic elements or genomic islands in the chromosome) on which T6SS genes are encoded (Fig. 3b). In this section, we discuss this variation in genomic environment as a factor that may explain the presence/absence variation. First, we focus on T6SSs that are encoded on genomic islands on the chromosome, and thereby were probably originally acquired by HGT and transmitted through inheritance (Fig. 3b, e). One example is the highly conserved pathogenicity island with T6SS genes in the causative agent of zoonotic diseases, *Francisella tularensis* and *Francisella novicida* [83–86]. Whereas *F. tularensis* and *F. tularensis* subsp. *holarctica* LVS encode two copies of the island, *F. novicida* has only one copy [87]. Similarly, T6SS-encoding *Salmonella* pathogenicity islands (SPIs) have been described in many *Salmonella* species carrying up to five phylogenetically distinct T6SS loci [88, 89]. A distinct type i1 T6SS was identified in a previously unreported pathogenicity island-like structure in some but not other *Salmonella bongori* strains. One strain carries another T6SS in SPI-22, which is common to all *S. bongori*, while the newly discovered T6SS bears a resemblance to the SPI-19 of *Salmonella* subgroup I lineages [90]. Differences in GC content between T6SS genes and the core genome have been noted across T6SS-positive bacteria [91–93], lending strength to the argument that these genes were acquired horizontally at some time point in the past. T6SSs encoded on genomic islands seem to be stably inherited by lateral gene transfer, with some intraspecific presence/absence variation.

Genes of the T6SS apparatus can be encoded on plasmids, lending them potential for exchange and spread by HGT (Fig. 3b). Examples include a plasmid of *Cronobacter* that occurs with (pESA3) and without (pCTU1) T6SS genes [94] and the recently described *Neisseria cinerea* 346T plasmid [95]. Some T6SS-carrying plasmids are very large and resemble chromosome-like structures. For instance, the T6SS-carrying plasmid pCJDM202 of *Campylobacter jejuni* strain WP2-202 is a megaplasmid with a size of over 110 kb. It is found in multiple *Campylobacter* species strains [22]. Similarly, some but not all *Pantoea ananatis* strains carry large pantoea plasmids, which encode T6SS genes and can reach sizes of over 700 kb [96]. Another T6SS-carrying plasmid is pRL12, one of six large plasmids of *Rhizobium leguminosarum* (*Rlv*), which has a multipartite genome, with large portions of its genetic material encoded on the plasmids. However, pRlvA, a plasmid very similar to pRL12, does not encode T6SS genes [97]. Such findings serve as indications for the movement of T6SS genes between the plasmid and the chromosome and the movement of T6SS-encoding plasmids between bacteria. Two *Campylobacter* species provide direct evidence for T6SS distribution and acquisition by mobile plasmids. *C. jejuni* and *Campylobacter coli* encode a complete set of T6SS genes in their chromosomes. These chromosomal T6SS gene clusters have a similar genetic organization and high relatedness to the T6SS genes in the plasmid pCJDM202, suggesting a common ancestor for the plasmid- and chromosome-encoded T6SSs [22]. Proof-of-principle experiments in the laboratory have shown that T6SS gene transfer between bacteria via plasmids is indeed possible. Marasini *et al*. demonstrated the gain of a (plasmid-encoded) T6SS by a formerly T6SS-negative strain by conjugation and showed that the newly T6SS-positive strain gained cytotoxicity towards red blood cells [22]. Similarly, Bruto *et al*. demonstrated transfer of a T6SS-positive plasmid to a T6SS-negative *V. crassostreae* strain and observed an increased virulence against the host oysters upon plasmid gain [17]. Phylogenetic analyses of *V. crassostreae* strains with and without the plasmid suggests multiple independent plasmid acquisition events (Fig. 2c, [80]). Thus, plasmids serve as a source of T6SS genes for bacteria and a means of T6SS gene distribution by lateral and horizontal gene transfer.

Genes of the T6SSs can be encoded on integrative and conjugative elements (ICEs), with potential for frequent exchange and spread, even between different species (Fig. 3b d). One example is the ICE of *Bacteroides uniformis* and other *Bacteroides* species with T6SS genes (Fig. 3d). Three different T6SS-encoding ICEs have been reported in *Bacteroides*, each with its own genetic architecture [98]. Coyne *et al*. observed remarkably high nucleotide sequence identities among T6SS-encoding ICEs of various *Bacteroides* strains and species from gut microbiome samples, suggesting frequent and very recent transfer of

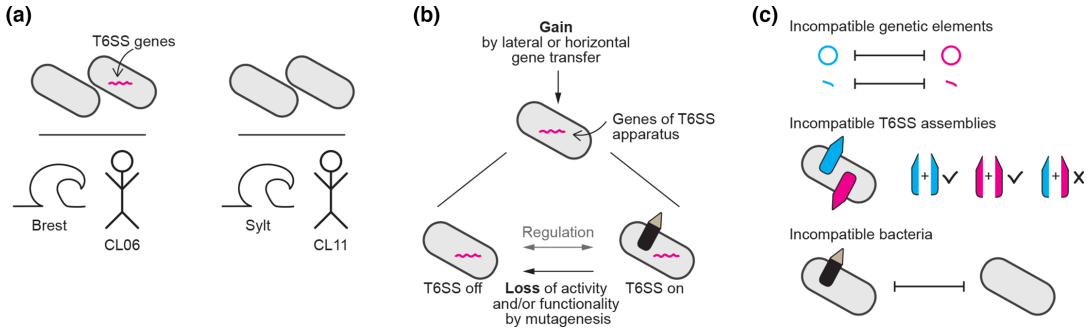


**Fig. 4.** Limits to T6SS spread. (a) Among two geographical locations and two individuals, T6SS-positive bacteria were found in mixed communities with T6SS-negative bacteria of the same species in one geographical location (Brest) and in the microbiome of one individual (CL06). In the second location (Sylt) and individual (CL11), no T6SS-positive bacteria were detected (right). Based on [60, 80]. (b) Depiction of the different molecular mechanisms underlying the gain and loss of T6SS genes. (c) Putative incompatibilities that might prevent the acquisition and accumulation of T6SSs in a bacterium.

T6SS genes [60]. These findings suggest that ICEs may be a means of exchange of T6SS genes between genomes, potentially at high rates, between *Bacteroides* species, although limited to this phylum.

## VARIATIONS IN GEOGRAPHICAL LOCATION AND CO-OCCURRENCE

Another factor that might affect T6SS presence/absence variation is the location and local environment of a given bacterium. T6SS-positive strains were found at different geographical locations and in different hosts from T6SS-negative strains of the same species. One example for T6SS-positive and T6SS-negative bacteria that are separated by geography is *V. crassostreae*, a marine species that harbours T6SS-carrying plasmids (Figs. 3c, 4a). Bruto *et al.* detected T6SSs in *V. crassostreae* isolates from Brest, near the Atlantic coast of north-west France, but not in isolates from Sylt in northern Germany [17], suggesting that strains carrying the plasmid were not selected for in the local environment of Sylt. An alternative explanation could be that the acquisition of the plasmid by HGT might have occurred rather recently in the Brest population, and the Sylt population has not acquired the plasmid because of the geographical separation. Another example for separation of T6SS-positive and T6SS-negative bacteria, not by geography but by host, is *B. uniformis* from the human gut microbiota (Fig. 4a). Whereas *B. uniformis* isolates with T6SS type GA1 were isolated from one individual (CL06), isolates from another individual (CL11) did not carry the T6SS type GA1 [60]. However, the T6SS GA1-negative isolates did carry another T6SS of a different type (as will be discussed in a later section). These examples share a common theme: a particular T6SS is detected among strains of a species from one site, but not among strains of the same species from another site, giving rise to a geographical or a host-mediated separation.

T6SS-positive and T6SS-negative strains of the same species have also been isolated from the same geography and host. In both examples mentioned above, the site from which T6SS-positive isolates were recovered also harboured T6SS-negative isolates. In other words, although all *V. crassostreae* isolates from Sylt were T6SS-negative, isolates from Brest included both T6SS-positive and T6SS-negative ones (Figs. 2c, 3a). Similarly, although all *B. uniformis* strains isolated from CL11 were T6SS-negative, isolates from CL06 included both T6SS-positive and T6SS-negative ones. This is noteworthy because T6SSs can be anti-bacterial weapons, and T6SS-negative bacteria might be killed by T6SS-positive bacteria, even if they are closely related, in a mixed population. The consequences of such mixed populations of T6SS-positive and T6SS-negative bacteria are further discussed in a later section of this review in the context of T6SS loss. Taking this and previous sections into account, factors such as the genomic environment (which determines the mobility of the T6SS genes) and geographical location might promote or inhibit the acquisition of T6SS genes and potentially contribute to the transition from a T6SS-negative to a T6SS-positive state. Equally relevant to T6SS presence/absence variation might be the opposite, the transition from a T6SS-positive to a T6SS-negative state through T6SS loss.

## ENERGETIC COSTS AND LOSS OF T6SSs

### Energetic costs of the T6SS

Expression of all the T6SS genes required for assembly and firing is an energetically expensive process. Each firing cycle requires the synthesis of up to 700 molecules of the Hcp tube proteins, which are exported, as well as the disassembly and preparation for reassembly of about 1500 TssB and TssC sheath proteins (Box 1) [99]. Given that TssB and TssC are recycled after the firing process (disassembly and restoration of the energy-rich state required for sheath contraction by the ATPase ClpV), it can be assumed that this is more energy-efficient than synthesizing TssB and TssC anew. We therefore use the cost of synthesizing all participating proteins as the upper limit of the energy expense for one firing event. Considering only the sheath and tube components, which make up the bulk of synthesized proteins, this would be about 700 Hcp (~160 aa per protein), 1500 TssB (~170 aa) and 1500 TssC (~500 aa)

proteins [99], adding up to slightly more than a million amino acids per firing event (or the equivalent of four million ATP energy bonds, assuming an energy expense of four ATP per added amino acid). For one firing event per minute, this upper limit for the energy expense would correspond to an average of about 50–100 synthesized proteins per second. This energy expenditure is similar to that for other secretion systems such as the type III secretion system (T3SS) [100]. The activity of the T3SS significantly slows down bacterial growth [101–104], and it is reasonable to believe that the same is true for the T6SS. However, although this assumption was included in models [26, 27], it has not yet been experimentally proven.

Regardless, the energy expenditure for operating the T6SS is considerable, and bacteria have evolved different strategies that mitigate these costs. As mentioned earlier, the high energy state of the non-secreted sheath proteins TssB and TssC can be restored [105–107]. In addition, *V. cholerae* T6SS proteins secreted into kin cells (Hcp, VgrG2 and effectors) are reused for the next assembly [108]. Alternatively, T6SS-positive strains can maximize the efficiency of the system by utilizing firing strategies or regulatory mechanisms to prevent unnecessary T6SS attacks and conserve energy, as bacteria do with other energy-expensive molecular mechanisms such as the T3SS and flagella. The ability to sense the direction of an incoming assault will also allow the T6SS counterattack to specifically aim at the target, in contrast to random firing, which will have a lower hit-to-miss ratio. Unlike constitutive T6SS expression such as in *V. cholerae* [1, 109] or *Serratia marcescens* [110], sensing incoming attack before activation of the T6SS has been shown to improve energy efficiency in *P. aeruginosa* [26]. Despite these cost-mitigation strategies, conditions might arise in which carrying a T6SS becomes too costly (because of energy expenses or other forms of costs), and bacteria without a T6SS might be favoured.

### T6SS loss by mutations

Some bacterial isolates carry mutations that result in an inactive or dysfunctional secretion apparatus (Fig. 4b). Perault *et al*. recently reported mutations in regulatory genes that abrogate T6SS activity in clinical *P. aeruginosa* isolates [19]. In *V. cholerae* strains from early pandemics, Kostiuk *et al*. described mutations in structural T6SS genes that inactivate the T6SS [21]. Frameshift mutations and truncations have been reported in genes essential for a functional T6SS in *Bacteroidales* [60]. Whether these mutations arose by genetic drift or by natural selection remains unknown. It is of note that isolates with such mutations are found under specific conditions. The T6SS-abrogating mutations in *P. aeruginosa* were detected in clinical isolates from patients with late-stage, but not early-stage, chronic lung infections. *V. cholerae* with the mutated T6SS genes were isolated from the second and sixth pandemics, but not the seventh pandemic. The mutated T6SS genes in *Bacteroidetes* were found exclusively in strains that carried two distinct types of T6SSs. Such examples may provide hints about conditions that favour a shift from a T6SS-positive to a T6SS-negative state, such as various forms of incompatibilities.

### Incompatibilities that might drive T6SS loss or prevent its acquisition and accumulation

Some bacteria, such as *P. aeruginosa* and *B. thailandensis*, carry and use multiple different T6SSs [61, 81]. Others harbour multiple T6SS gene clusters, although only one is functional. The latter occurs in *Bacteroidetes* and is a strong indication for T6SS exclusion, a process which prevents or selects against the acquisition of a functional T6SS [60]. Based on this example, we will speculate more broadly about three mechanisms that might prevent T6SS acquisition and accumulation of multiple T6SSs (Fig. 4c). First is incompatibility between mobile genetic elements that encode T6SS genes and mobile elements already present in the recipient bacterium. In the case of *Bacteroidetes*, the T6SS is encoded on ICEs that can be transferred horizontally, both within and between species [60]. ICE exclusion is a known phenomenon in Gram-negative and Gram-positive bacteria, where the presence of an ICE in a bacterium prevents the acquisition of the same or similar ICEs [111, 112]. However, the GA1- and GA2-carrying ICEs in *Bacteroidetes* show low sequence conservation, making this explanation unlikely in this particular case. The same idea applies to plasmid incompatibility that could prevent the acquisition of a plasmid with T6SS genes. Second, incompatibility in the assembly of multiple T6SSs might prevent T6SS accumulation. If the recipient bacterium already has a T6SS and acquires a second, slightly different, one, the two T6SSs could hamper the assembly of each other by forming dysfunctional multimers that do not form a functional secretion apparatus. So far, there is no experimental evidence for dysfunctional chimaeras of various T6SSs. Indeed, some examples demonstrate rather cases of structural components being shared between T6SSs [108, 113] or of multiple T6SSs coexisting in the same bacterium [114]. Notably, a comparison with other secretion systems shows that bacteria with multiple T3SSs do not typically express them simultaneously, but under different conditions [115]. Third, incompatibility between donor and recipient bacterium might prevent the uptake of T6SS genes. Given that type VI secretion is a killing mechanism, a T6SS-negative (potential) recipient might be killed by the T6SS of the (potential) T6SS-positive donor before conjugative transfer could occur, especially if direct contact is necessary for conjugative transfer of T6SS-encoding mobile genetic elements. This mechanism has in fact already been reported, albeit in a different context: Venanzio and colleagues reported that T6SS-mediated killing of T6SS-negative recipients by T6SS-positive donors prevented the spread of antimicrobial resistance genes [70]. Although bacterial incompatibility and subsequent killing has been reported to foster DNA uptake by HGT when linked to natural competence in *V. cholerae* [116–118], we are so far not aware of any indication for the uptake of entire T6SS gene clusters via this mechanism. Taken together, various incompatibilities might prevent the acquisition of T6SS genes or favour an inactive T6SS upon acquisition.

### Particularities of losing a secretion system involved in bacteria–bacteria killing

A factor that requires careful consideration when speculating on T6SS loss is the role of the T6SSs in bacteria–bacteria interactions and their functions in bacterial killing (Box 1). While loss of the T6SS in a solitary bacterium that is not part of a community might have little or no direct consequence, loss of the T6SS in a bacterial community may directly impact the survival of the bacterium, as it could become susceptible to attacks from neighbouring T6SS-positive siblings or competitors. One possibility to avoid this fate is to retain the immunity protein-encoding genes that mediate protection from incoming toxic T6SS effectors of attacking bacteria, while disposing of the T6SS machinery itself. In some bacteria, so-called orphan immunity genes can be detected independent of the T6SS locus that seem to serve the purpose of protection from an external attack [119–121]. If T6SS-negative (but immune) bacteria gain a fitness advantage, this genotype could gradually become dominant in the resulting mixed communities, which in turn would allow for the complete loss of all T6SS-associated genes including the immunity proteins. This evolutionary route would be favoured in species where immunity proteins are encoded and active independently of the apparatus, such as *V. cholerae*, and less likely in species where immunity proteins and structural proteins are co-regulated, such as in *Burkholderia* [122]. Means of protection other than immunity proteins might further decrease the need for a functional T6SS [123, 124]. In competition with other bacteria, T6SS-positive and T6SS-negative (but immunity-positive) siblings could exercise division of labour, for example by bimodal expression of the T6SS machinery proteins. The resulting populations would benefit from both the ability to kill competitors and faster division through improved energy efficiency. While such strategies have been observed in the case of the T3SS [104], they have been considered, but not yet directly described in the case of the T6SS [125]. Moreover, T6SS is a contact-dependent mechanism, which can lead to a physical barrier of dead cells at the interface of T6SS-positive and T6SS-susceptible populations. This could limit the advantage of having a T6SS to the point where T6SS-negative populations that are partially protected by this mechanism can outgrow their T6SS-positive competitors [27, 125–127].

## CONCLUSIONS AND FUTURE CHALLENGES

T6SS-positive bacteria are phylogenetically diverse and use the system to engage in interactions with their biotic and abiotic environment. However, it is clear that those bacteria without a T6SS apparatus might not necessarily be worse off, depending on the context. To explain the observed presence/absence variation of T6SS genes, we may invoke a combination of ultimate causes (such as adaptations to reduce the energetic costs of carrying a T6SS) and proximate causes (such as the mechanisms of T6SS gain and loss through HGT). In some species, having or not having the T6SS might not resemble two opposing poles, but rather a dynamic continuum, in which bacteria transition from one state to another (i) by acquiring T6SS genes via HGT or (ii) by losing T6SS genes through mutations or gene loss, in addition to differential regulation of T6SS genes, as has been covered in existing reviews [37, 38, 40, 42–44, 128, 129].

Speculating on the benefits of having a T6SS is hardly possible without at least considering the effectors, which can have very diverse functions and differ between bacteria [32, 92]. For many of the species discussed in this review, the effectors and their activities remain unknown. Knowing more about these effectors in the future will also help us to better understand the presence/absence variation of the secretion system that exists to translocate these effectors. The unique capability of the T6SS machinery to translocate effectors into prokaryotic, eukaryotic and fungal cells or to the extracellular space makes the discovery of more T6SS-mediated functions very likely.

A better understanding of the different T6SS subtypes might also improve our understanding of the spread of the secretion apparatus across microbes. The differences in prevalence of T6SS$^{ii}$ and T6SS$^{iii}$, which are specific to individual phyla, and the more widespread T6SS$^{i}$ and T6SS$^{iv}$ are striking. Whereas T6SS$^{i}$ and T6SS$^{ii}$ are more or less well understood, comparatively little is known about T6SS$^{iii}$ and T6SS$^{iv}$, underlining the need for more comparative work that encompasses all subtypes. The resemblance of the T6SS to phage tails [130–132] and particularly the close relatedness of T6SS$^{iv}$ to extracellular contractile injection systems [13, 14] raise the question of whether T6SSs arose from a common ancestor or from multiple transition events. Deciphering the early traces of T6SS evolution might provide new insights into the spread of T6SS genes across bacterial populations and various forms of adaptation of the secretion apparatus to the lifestyles of particular microbes.

Further work is needed to fully grasp the prevalence of the T6SS among bacteria. In some phyla, the system has only been identified at the genome level, and awaits functional characterization. Among species with well-studied T6SSs and intraspecific presence/absence variation, extended sampling of natural populations will provide more insights into environments that favour T6SS presence or absence. For comparative studies at large scales, less bias would be imposed by a more comprehensive collection of genome sequences. Additional longitudinal sampling will allow for assessment of dynamics over time. Future studies on such focus areas will probably provide some answers, and are likely to raise more questions on the presence and absence of the T6SS.

Funding information
R.U. is funded by the International Max-Planck Research School for Evolutionary Biology (IMPRS EvolBio). Work in the Diepold Lab is supported by the Max Planck Society and the German Research Foundation (grant DI 1765/5-1). Work in the Unterweger Lab is supported by the German Research Foundation (grant CRC1182) and the German Federal Ministry for Education and Research (grant 01KI2020).

Acknowledgements
We thank Antonia Habich for support with bioinformatics and all members of the Unterweger Lab for discussion and comments.

Author contributions
All authors contributed to the writing of the original draft, and reviewed and edited the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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