Diversity And Distribution of Type VI Secretion System In Bacterial Plasmids

Sergio Morgado (sergio.morgado@ioc.fiocruz.br)
Oswaldo Cruz Institute
Ana Carolina Vicente
Oswaldo Cruz Institute

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Abstract

Type VI Secretion System (T6SS) is a nanomolecular apparatus that allows the delivery of effector molecules through the cell envelope of a donor bacterium to prokaryotic and/or eukaryotic cells, playing a role in the bacterial competition, virulence, and host manipulation. T6SS is patchily distributed in bacteria and rarely reported in mobile elements, such as plasmids and integrative and conjugative elements. The impact that T6SS may have on bacteria fitness and the lack of evidence on its radiation mechanism led us to question whether natural plasmids could represent a key mechanism in the spread of T6SS in bacteria. Therefore, we performed an *in-silico* analysis to reveal the association of T6SS and plasmids. This secretion system was mined in more than 30,000 plasmids from Genbank, based on the presence of at least nine of 11 T6SS core proteins (TssA-M). T6SS was identified in 303 plasmids (~1%), all belonging to the same T6SS type, occurring mainly in *Proteobacteria* (301/303), particularly in *Rhizobium* and *Ralstonia* genera. Interestingly, most of the bacteria carrying T6SS-harboring plasmids did not encode T6SS in their chromosomes, suggesting a fitness cost to maintain this element on mobile platforms.

Introduction

Microbial communities are dynamic due to the myriad interactions of their members. In these communities, bacteria can communicate with their surrounding through the type VI secretion system (T6SS). This nanomolecular apparatus allows the delivery of effector molecules through the cell envelope of a donor bacterium to prokaryotic and/or eukaryotic cells, playing a role in the bacterial competition, virulence, and host manipulation [1–3]. This secretion system is assembled by several proteins (TssA-M), which make up a membrane complex, baseplate, syringe, needle spike, and sheath [2, 4–5]. Currently, T6SS is classified into four types (i, ii, iii, iv) based on the diversity of TssB protein sequences and has distinct genetic architectures [4]. Most T6SSs belong to type i, which is prevalent in *Proteobacteria*, and so far, is classified into six subtypes (i1, i2, i3, i4a, i4b, and i5). T6SS types ii and iii were found exclusively on *Francisella* pathogenicity islands and *Bacteroidetes*, respectively; while type iv was observed in *Amoebophilus* [6, 7].

The lack of ubiquity and the diversity of T6SSs in the chromosome of a wide variety of genera suggest that some T6SS clusters are more likely to be acquired by horizontal gene transfer. Furthermore, some bacteria harbor more than one T6SS of different phylogenetic types, which also suggests acquisition by horizontal gene transfer [4, 8, 9]. However, in contrast to this evidence, T6SSs are rarely reported in association with mobile elements such as plasmids and integrative and conjugative elements [2]. Therefore, this scenario led us to question whether the distribution of this secretion system has been underexplored in plasmids and whether T6SS radiated and evolved ancestrally through plasmids within bacteria, in the same way as was inferred for the type VII secretion system (T7SS) in *Mycobacteriaceae* [10]. Thus, we performed an *in-silico* analysis to characterize the T6SS in all plasmids available from Genbank. We observed a limited distribution of T6SS in the thousands of analyzed plasmids. Most of the

T6SS-harboring plasmids were harbored by environmental *Proteobacteria*. Interestingly, most bacteria carrying T6SS-harboring plasmids did not encode T6SS on their chromosomes.

Results

Characterization of T6SS-harboring plasmids

We performed an *in-silico* analysis to reveal the association of T6SS and plasmids. We mined T6SS on over 30,000 plasmids from Genbank based on the presence of at least nine of the 11 T6SS core proteins (TssA-M). In this way, T6SS was identified in 303 plasmids (~1%) with lengths ranging from 34 kb to 2.8 Mb (1.6 Mb median) and GC content from 38–72% (63% median) (TableS1). Most of these 303 plasmids were characterized as non-mobilizable (n=211), while the rest as conjugative (n=61) and mobilizable (n=31). These T6SS-harboring plasmids were distributed in 280 genomes of 22 bacterial families and three phyla, *Acidobacteria*, *Gemmatimonadetes*, and *Proteobacteria* (Table 1 and TableS1). Within the phylum *Proteobacteria*, T6SS-harboring plasmids were prevalent in two classes, α-*Proteobacteria* (n=112) and β-*Proteobacteria* (n=140) (TableS1). Considering genera with at least 10 T6SS-harboring plasmids, *Ralstonia* had the highest relative abundance (~80%), while *Rahnella* had 47%, and the others had less than 14% of relative abundance (TableS1 and TableS2). Most bacteria carrying T6SS-harboring plasmids have been isolated from the environment, including roots, soils, water, seeds, plants, foods, while few have been isolated from humans or animals (TableS1). We also investigated the presence of T6SS in the chromosome of the 280 bacteria carrying T6SS-harboring plasmids and verified T6SS in 50 (~17%) chromosomes.

Table 1
Features of T6SS-carrying plasmids

Families	Number of plasmids	Median size (kb)	Median GC	Prevalent T6SS type
Acidobacteriaceae	1	475	0.6	i4b
Aeromonadaceae	1	34	0.56	i1
Aurantimonadaceae	1	488	0.68	i5
Azospirillaceae	14	1,761	0.69	i4a
Burkholderiaceae	140	2,027	0.67	i4b
Enterobacteriaceae	13	171	0.53	i2
Erwiniaceae	8	317	0.52	i2
Gemmatimonadaceae	1	1,106	0.73	i4b
Hafniaceae	1	145	0.47	i4b
Halomonadaceae	1	1,833	0.55	i1
Moraxellaceae	1	127	0.41	i3
Phyllobacteriaceae	7	531	0.6	i5
Pseudoalteromonadaceae	3	899	0.41	i5
Pseudomonadaceae	1	371	0.55	i1
Rhizobiaceae	69	655	0.59	i3
Rhodobacteraceae	13	215	0.67	i3
Rhodospirillaceae	1	692	0.68	i5
Roseobacteraceae	5	148	0.62	i3
Sphingomonadaceae	1	2,710	0.63	i3
Thalassospiraceae	1	908	0.54	i1
Vibrionaceae	10	1,504	0.45	i1
Yersiniaceae	10	554	0.52	i4b

In a loose analysis, considering plasmids with less than nine T6SS genes, we observed 327 plasmids belonging to several phyla, in addition to *Proteobacteria*: *Bacteroidetes*, *Spirochaetes*, *Firmicutes*, *Deinococcus-Thermus*, *Cyanobacteria*, and *Actinobacteria*. Considering plasmids with exactly eight T6SS genes (the number closest to the previous strict selection threshold), 21 *Proteobacteria* plasmids were observed, 15 of which belong to *Campylobacter* (class ε-*Proteobacteria*), a genus absent in the strict

analysis. Furthermore, in this loose analysis (considering those with two to eight T6SS genes), 60/93 *Proteobacteria* plasmids with T6SS were in γ-*Proteobacteria*. Curiously, all non-*Proteobacteria* plasmids harbored only one T6SS subunit, TssE.

As T6SS is also associated with other mobile elements, such as genomic islands, we explored some features related to these elements in the plasmids. In fact, 110/303 of them had transposase genes surrounding the T6SS regions. Another indication of T6SS horizontal transfer acquisition could be discrepant levels of GC content between the T6SS region and the rest of the plasmid, but in general, no significant differences were observed, with the largest differences around 5%.

Plasmid T6ss Classification

The T6SS classification scheme, based on the sequence of the TssB component (VipA or IglA), showed that all T6SS harbored by the 303 plasmids belonged to the type i, with a prevalence of i4b and i3 subtypes. A maximum-likelihood tree, based on these TssB sequences, showed the clustering of the different subtypes, each with related groups of taxa (Figure 1). There was no association of a T6SS subtype with a specific taxon, since different subtypes were identified in the same taxon, such as *Rhizobium* (i1, i3, and i5), *Rahnella* (i1, i2, and i4b), *Paraburkholderia* (i2, i3, i4a, and i4b), and *Azospirillum* (i1, i4a, and i5). Curiously, the two non-*Proteobacteria* sequences (*Acidobacteria* and *Gemmatimonadetes*) clustered in the same clade in a branch of the i4b subtype. Almost all defined clusters contain chromosomal reference sequences, with no clear separation of chromosomal and plasmid sequences, suggesting that there is no independent evolution in these genetic compartments. Interestingly, there is a cluster with sequences from different genera (e.g., *Paracoccus*, *Mesorhizobium*, *Sinorhizobium*, *Rhizobium*, *Rhodobacter*), plasmid sizes (~267 kb - 2.4 Mb) and classified as subtype i3 that was positioned apart from other sequences of the i3 subtype (Figure 1, red branch), which could represent a new T6SS subtype, until now, plasmid-exclusive T6SS subtype. This diversity of subtypes is reflected in their genetic neighborhood, as can be seen by the differences in the genetic architecture of each subtype (Figure 2).

Gene Content Of T6ss-harboring Plasmids

As T6SS provides fitness and colonization advantages, we also searched for other plasmid cargo genes, such as T6SS effectors, virulence, antibiotic resistance, and secondary metabolites. Regarding the presence and type of T6SS effectors, of the 303 plasmids, 208 encoded 261 effectors, ranging from one (majority) to four effectors. These effectors belonged to seven types, *hcp* (n=204), *vgr*G (n=32), *mod*A (n=9), PAAR-like (n=6), polymorphic toxin (n=3), Ig-like and *tag*O (n=1), plus some unnamed effectors. The search for virulence and antibiotic resistance genes in these plasmids revealed that 7/303 and 21/303 encoded genes associated with antibiotic resistance and virulence (disregarding T6SS genes), respectively (TableS3 and TableS4). Plasmids with more antibiotic resistance and virulence genes were mainly associated with bacteria recovered from human or animal hosts. Interestingly, it was identified in

126 plasmids, gene clusters with 100% similarity to 10 metabolite types of non-ribosomal peptide synthetase (NRPS), ectoine, and terpene (TableS1). These metabolites were associated with siderophores, osmotic protection, photosynthesis, antimicrobial and antifungal activities; and each type of metabolite gene cluster was only found on plasmids of specific genera, e.g., ralsolamycin and rhizoxin in *Ralstonia*, vicibactin in *Rhizobium*, and carotenoid in *Pantoea* (TableS1). Curiously, hundreds of these plasmids had genes associated with protein synthesis, such as rRNA (n=115) and tRNA (n=191).

Discussion

T6SS is a bacteria strategy to compete for a niche. The gene clusters of this system are often located in genomic islands, which have the potential to be transferred, as a unit, to other cells. To date, T6SSs have been identified in several genera of six phyla of Gram-negative bacteria, *Acidobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, *Nitrospirae*, *Planctomycetes*, and *Proteobacteria*, being abundant in the latter [6, 9, 11, 12]. This wide (but not ubiquitous) distribution and diversity of T6SS in different genera of these phyla suggest an association of T6SS with horizontal gene transfer. However, so far, only twenty-nine plasmids with T6SS had been reported [2]. Even so, despite being in mobile elements, plasmid T6SSs can be functional [13–15].

Here, we mined hundreds of T6SS-harboring plasmids, and in dozens of cases, the bacteria did not have chromosomal T6SS, indicating and reinforcing the role of carrier elements, such as plasmids, in the dispersion of this secretion system. Furthermore, although present in the chromosome of several phyla and genera [11], indeed, the distribution of T6SS in plasmids is limited, as only ~1% of them encoded this secretion system, mainly *Proteobacteria*. Several factors may contribute to this phenomenon: (i) the dissemination of T6SS via plasmids, at least in *Proteobacteria*, seems to have barriers, since bacteria with chromosomal T6SS (abundant in *Proteobacteria*) may present a defense mechanism via T6SS against the acquisition of new plasmids [2]; (ii) carrying an extra copy of T6SS does not seem advantageous if the bacterium already has a chromosomal copy, as it is a niche-specific system and different T6SSs do not confer different functions, depending more on the effectors that are secreted [4]; (iii) since T6SS-harboring plasmids have a large median size (1.6 Mb), this would likely impose a high fitness cost. Previously, Abby et al., (2016) showed that chromosomal T6SS was more prevalent in γ-*Proteobacteria* than in α- and β-*Proteobacteria*, and curiously, here, we observed that the plasmid T6SS prevail in α- and β-*Proteobacteria*. On the other hand, plasmids with T6SS were prevalent in γ-*Proteobacteria* when considering those carrying less than nine T6SS genes, which suggests that smaller clusters of T6SS would be more common in γ-*Proteobacteria* plasmids and that once these elements are stabilized in the chromosome, the T6SS plasmid copy would undergo a process of degradation and eventually lost, which could result in a low prevalence of T6SS in such mobile elements. Furthermore, in the loose analysis, several non-*Proteobacteria* plasmids harbored a T6SS gene, TssE, which is homologous to the bacteriophage T4 gp25 baseplate [16]. Thus, it is likely that these non-*Proteobacteria* plasmids are not related to T6SS. Here we also observed T6SS in a few plasmids from other phyla, in addition to *Proteobacteria*, *Acidobacteria*, and *Gemmatimonadetes*, which may indicate their acquisition from other bacteria in the environment.

Of the four phylogenetic T6SS types, type i was the only one found in plasmids. Furthermore, this type is the most common in *Proteobacteria* [11]. This scenario is evidence that plasmids have irradiated T6SSi, at least, in *Proteobacteria*. Looking at our dataset and the T6SS subtypes, types are prevalent i and ii on chromosomes, while types i4b and i3 prevail on plasmids. Of note, there was a subcluster in the subtype iii cluster with dozens of plasmids sequences that were not closely related to any reference chromosomal sequence. Most of these sequences belonged to environmental bacteria and could be evolving independently of the others of subtype iii.

Considering the gene cargo of the analyzed plasmids, we did not observe in most of them a prevalence of resistance or virulence genes (disregarding the T6SS). Thus, unless these T6SSs play a virulence role in their host niche, these plasmids would be more related to some ecological role. Even because some of them also encode secondary metabolites related to survival and protection. Among the T6SS effectors identified in the plasmids, most of them would be related to virulence, however, *mod*A is associated with nitrate metabolism and anaerobiosis, being an important element for plant-associated bacteria [17]. Interestingly, this effector was found only in *Azospirillum* plasmids (9/13). These ecological gene cargos contrast with virulent T6SS-harboring plasmids from clinical bacteria, such as *Cronobacter* spp. and *Campylobacter jejuni* [18, 19]. The few plasmids identified carrying resistance and virulence genes were mainly associated with bacteria isolated from human or animal hosts. Indeed, clinical T6SS-positive bacteria were observed to have a higher resistance and frequency of virulence genes [20]. Although 92/303 of these T6SS-carrying plasmids have been characterized as conjugative or mobilizable [21], their median size (~601 kb) would represent a natural restriction to transmission. Thus, these mobility genes may be part of integrative elements, such as integrative conjugative elements (ICEs) [22]. Indeed, ICEs have been predicted in some plasmids characterized as conjugative (e.g., NZ_CP064859.1, NZ_CP057783.1, NZ_CP017887, and NZ_CP053574.1). Furthermore, recently, it was shown in *Bacteroidales* that T6SS presents an extensive intra-ecosystem transfer and multi-species spread due to its association with ICEs [23]. Therefore, in addition to plasmids, other mobile platforms, such as ICEs, may be involved in the spread of T6SS. Finally, in dozens of these T6SS-harboring plasmids we identified genes associated with chromosomes, such as rRNA, and this, added to the fact that most of them are megabases in size, raised the question of whether they were in fact plasmids or another type of replicon. In fact, some of the genera identified here were associated with secondary essential replicons (secondary chromosomes or forming chromosomes), such as *Burkholderia*, *Cupriavidus*, *Ensifer*/ *Sinorhizobium*, *Pantoea*, *Ralstonia*, *Rhizobium*, *Vibrio* [24].

Therefore, our findings do not fully support the hypothesis that T6SS irradiation within bacteria was plasmid-mediated, as occurred with T7SS in *Mycobacteriaceae* [10]. Even so, the evidence gathered here points to the involvement of mobile platforms in the spread of T6SS within bacteria.

Methods

Plasmids analyzed

A total of 30,660 plasmids were obtained from NCBI in Sep-2021: https://www.ncbi.nlm.nih.gov/genome/browse/#!/plasmids/, encompassing more than 20 bacterial phyla (TableS2) and annotated using Prokka v1.12 [25].

T6ss Identification, Classification, And Phylogeny

The T6SS core proteins were searched through the proteomes of the 30,660 plasmids by the hmmsearch program [26] using hmm profiles of 11 Clusters of Orthologous Groups of proteins (COGs) listed in Table 2. Plasmids that encoded at least nine of the eleven T6SS core proteins were considered carriers of T6SS. We considered 11 COGs instead of 13, as COG3501 (VgrG) and COG0542 (ClpV) were shown not to be T6SS specific [5]. The classification of plasmid-borne T6SSs was based on the TssB component sequence, where for each T6SS, TssB sequences were submitted to SecReT6 web platform (https://bioinfo-mml.sjtu.edu.cn/SecReT6/phylogenetic_analysis.php) in T6SS classification tool [6]. Using these TssB sequences, a phylogeny of plasmid T6SSs was performed. Initially, the TssB sequences were aligned, and the low-quality alignment columns were removed using GUIDANCE2 v2.02 [27]. Then, the TssB alignment was submitted to IQTree v1.6.12 [28] to obtain a maximum likelihood tree, which used the model of substitution WAG+G4 and 1000 ultrafast bootstrap replicates [29]. The tree was visualized using the iTOL web platform (https://itol.embl.de) [30].

Table 2
List of T6SS core genes used

Protein	COG	synonym	Domain access
TssA	COG3515	impA/vasJ	PF06812
TssB	COG3516	impB, vipA	PF05591
TssC	COG3517	impC, vipB	TIGR03355.1
TssD	COG3157	Hcp	PF05638
TssE	COG3518	mpF, vasS	PF04965
TssF	COG3519	impG, vasA	PF05947
TssG	COG3520	impH, vasB	PF06996
TssJ	COG3521	vasD, lip	PF12790
TssK	COG3522	impJ, vasE	PF05936
TssL	COG3455	ompA/dotU	PF09850
TssM	COG3523	vasK, icmF	PF06744

Characterization Of T6ss-carrying Plasmids

Plasmids considered T6SS carriers were characterized concerning their gene cargo: clusters of secondary metabolites were mined using antiSMASH v6 [31]; virulence and antibiotic resistance genes were screening by ABRicate (https://github.com/tseemann/abricate) based on VFDB [32] and CARD [33] databases (Sep-2021); T6SS effectors (T6SEs) were identified within and flanking the T6SS regions using BLAST considering 50% identity and 60% coverage. The queries consisted of 294 experimentally verified T6SEs from the integrated database SecReT6 [6] (Sep-2021).

Declarations

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Author contributions

Conceptualization, A.C.V.; methodology, S.M., and A.C.V.; formal analysis, S.M.; writing—original draft preparation, S.M., and A.C.V.; writing—review and editing, S.M., A.C.V.; supervision, A.C.V. All authors have read and agreed to the published version of the manuscript.

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Figures

Figure 1

Phylogenetic relationship of T6SS plasmid clusters based on TssB core component protein and maximum likelihood method. The sequences are divided into four types and six subtypes by colored backgrounds. Chromosomal reference sequences are marked in red. The red branch represents sequences assigned to the i3 subtype, but which are not grouped with the i3 reference sequences. The mobility of the plasmids that harbor the T6SS clusters is indicated externally. Bootstrap values above 70 are shown as red circles in the middle of the branches.

Figure 2

Genetic architecture of T6SS clusters of different type i subtypes.

Supplementary Files

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