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SecReT6 update: a comprehensive resource of bacterial Type VI Secretion Systems

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Type VI Secretion System (T6SS) plays significant roles in microbial activities via injecting effectors into adjacent cells or environments. T6SS increasingly gained attention due to its important influence on pathogenesis, microbial competition, etc. T6SS-associated research is explosively expanding on numerous grounds that call for an efficient resource. The SecReT6 version 3 provides comprehensive information on T6SS and the interactions between T6SS and T6SS-related proteins such as T6SS regulators and T6SS effectors. To assist T6SS researches like microbial competition and regulatory mechanisms, SecReT6 v3 developed online tools for detection and analysis of T6SS and T6SS-related proteins and estimation of T6SS-dependent killing risk. We have identified a novel T6SS regulator and T6SS-dependent killing capacity in *Acinetobacter baumannii* clinical isolates with the aid of SecReT6 v3. 17,212 T6SSs and plentiful T6SS-related proteins in 26,573 bacterial complete genomes were also detected, analyzed and incorporated into the database. The database is freely available at https://bioinfo-mml.sjtu.edu.cn/SecReT6/.

Type VI Secretion System, T6SS-related protein, database, prediction, *Acinetobacter baumannii*

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INTRODUCTION

Type VI Secretion System (T6SS) is a vital dynamic protein delivery apparatus that contributes to bacterial pathogenesis, competition, stress response, and metal acquisition (Basler et al., 2013; Si et al., 2017; Wang et al., 2019a; Weber et al., 2016; Zhu et al., 2021). T6SS consists of dozens of structural proteins, such as TssA−M, and a few T6SS accessory proteins (T6SAs), like TagA and TagL (Vettiger et al., 2017; Wang et al., 2019a). Based on the phylogenetic analysis of the sheath protein TssB, T6SSs are divided into four groups: T6SS^{i}, T6SS^{ii}, T6SS^{iii}, and T6SS^{iv}. T6SS^{iv} is newly discovered and represents a primordial system from which extracellular contractile injection systems, phages, and T6SS$^{i–iii}$ have evolved (Böck et al., 2017; Russell et al., 2014).

T6SS operates via expelling a broad range of T6SS ef-

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fectors (T6SEs) into target cells or extracellular environments (Basler et al., 2013; Coyne and Comstock, 2019; Si et al., 2017; Weber et al., 2016). T6SEs are divided into specialized effectors and cargo effectors. Specialized effectors contain functional domains and a domain of TssD/TssI/PAAR. Cargo effectors are secreted via binding with TssD/TssI/PAAR directly or indirectly with the aid of adaptors in T6SA, such as DUF4125 and DUF1795 (Burkinshaw et al., 2018; Liang et al., 2015). T6SS immunity proteins (T6SIs) could specifically neutralize the toxicity of the cognate T6SE, and the genes coding for T6SI and T6SE are usually closely located (Basler et al., 2013; Liang et al., 2015). Recipient susceptibility factors are non-specific immune regulators or mechanisms in recipient bacteria to respond to T6SE attacks from exogenetic T6SS (Hersch et al., 2020; Lin et al., 2020). T6SS function is tightly regulated by various regulators (T6SRs) under complex mechanisms like the threonine phosphorylation pathway (TPP), quorum-sensing (QS), and two-component systems (TCSs). T6SSs are usually silenced and are influenced or switched on by certain changes in temperature, PH, second messenger molecule cyclic di-GMP, nutrient, or damage to the cell membrane (Chen et al., 2015; Ho et al., 2013; Joshi et al., 2017; Storey et al., 2020; Wang et al., 2019a).

The T6SS-associated information has increased exponentially, ranging from T6SSs to T6SS-related proteins, including T6SEs, T6SIs, T6SRs, T6SAs, T6SS component proteins, and recipient susceptibility factors. Studies on T6SS are expanding into various aspects of microbial research, including interaction in the microbial community, pathogenicity of clinical strains, horizontal gene transfer, and metal ion acquisition (Borgeaud et al., 2015; Fu et al., 2018; Si et al., 2017; Zhu et al., 2021). Several public databases and tools that involved T6SS research have been developed. SecReT6 (Li et al., 2015) original version collected the experimentally verified T6SS gene clusters, T6SEs and T6SIs. SecretEPDB (An et al., 2017) and EffectiveDB (Eichinger et al., 2016) have integrated effectors of bacterial multiple secretion systems. Recently, BastionHub (Wang et al., 2021) provided the collection of secretion substrates of types I–IV and VI secretion systems and effector prediction, which functions like Bastion6 (Wang et al., 2018). These databases mainly focused on the collection of effectors from different secretion systems, lacking support for deeper mining of T6SS comprehensive information, such as the incredible amount of data on T6SS function and classification (Böck et al., 2017; Le et al., 2021), T6SS-related proteins and their interactions (Bernal et al., 2021; Burkinshaw et al., 2018), and T6SS regulation (Joshi et al., 2017; Storey et al., 2020). The key protein delivery system T6SS, widespread in Gram-negative bacteria requires a specific and detailed database.

Here, we report the new major release of SecReT6, version 3. It reflects an increase in the curated dataset of the known T6SSs, T6SEs, and T6SIs. In addition, the new version also includes recently collected T6SRs, T6SAs, and the recipient susceptibility factors with experimental support. T6SS-dependent killing risk estimation tool is provided to support studies on microbial antagonism. The newly developed tools for T6SS-related protein prediction and T6SS classification are also included. SecReT6 v3 contains 17,212 putative T6SSs and plentiful T6SS-related proteins in 26,573 bacterial genomes. With the support of SecReT6 v3, a new negative T6SR was detected, and the *E. coli* killing capacity of T6SSs was characterized in *Acinetobacter baumannii* clinical isolates. We expect SecReT6 v3 will provide improved and reliable support for bacterial type VI secretion systems-related research.

RESULTS

Interaction and classification of T6SS and T6SS-related proteins

The overview of the SecReT6 v3 framework and content is shown in Figure 1. SecReT6 v3 provided interactive information of T6SS and T6SS-related proteins sorted in categories, including newly incorporated T6SAs and T6SRs, as well as updated T6SEs, T6SIs, and structural component proteins. Intuitive graphs presented the interaction of the T6SS and T6SS-related proteins in the T6SS detailed information interface, accessible by clicking T6SS ID links from the "Statistics" module or the "Browse" module. For instance, the connection of T6SA TecT and co-TecT, PAAR4, T6SE TseT, and H3-T6SS in *Pseudomonas aeruginosa* PAO1 (Burkinshaw et al., 2018) is presented by an intuitive directed network graph. T6SA TecT and co-TecT function as the bridge to secreted substrate PAAR4 of H3-T6SS and TseT. TecT is the common adaptor containing the DUF4123 domain, a T6SE marker, and can aid T6SE binding to T6SS secrete substrate PAAR/VgrG (Burkinshaw et al., 2018; Liang et al., 2015). All the interactions of T6SS-related proteins and T6SS were experimentally supported. Users can get detailed information on linked T6SS or T6SS-related proteins by clicking the interaction chart (Figure S1 in Supporting Information).

T6SS data were categorized in detail in the "Browse" module according to data type. Each data type including T6SS, T6SE, T6SR, and T6SA, is further classified according to its features. Data were visualized via pie charts or diagrams, with classification and description in subpages of Taxonomy and classification in the "Statistics" module. For example, in the classification subpage in the "Statistics" module, T6SEs were classified from Tle to Tie according to the T6SE function (Jurėnas and Journet, 2021; Si et al., 2017; Yadav et al., 2021). Tie was the first kind of T6SE we collected that was associated with metal ion acquisition, like

Figure 1 Framework and contents of bacterial Type VI secretion system database SecReT6 v3.

TseM (Si et al., 2017). T6SRs were categorized via literature-reported regulatory mechanisms or pathways from the KEGG database. All data were also presented according to replicon or taxonomy (Figure S2 in Supporting Information).

Case Study 1: Prediction and analysis of T6SSs in sequenced bacterial genomes

SecReT6 v3 provides a T6SS prediction tool publicly available online and as a standalone version. We have detected 17,212 T6SSs in 26,573 complete bacterial genomes with the feature of "Complete" or "Chromosome" from NCBI. According to the result, at least one T6SS was present in the individual bacterial pathogens, such as 217 (74.6%) out of 291 *Acinetobacter baumannii* strains, 376 (96.4%) out of 390 *Pseudomonas aeruginosa* strains, 851 (92.9%) out of 916 of *Klebsiella pneumoniae* strains and 116 (100%) out of *Serratia marcescens* strains (Table S1 in Supporting Information), which revealed the importance of T6SS to these clinical pathogens. We also found 17 new classes in bacteria containing putative T6SSs such as those in *Planctomycetia* and *Negativicutes*, which need further experimental validation. All predicted T6SSs were clustered and separated into four classes, including nine sub-classes, in the same way as the experimental supported T6SSs collected by SecReT6 v3 (Figure 2). For example, out of 116 complete genomes of *Serratia marcescens*, 47 (40.5%) strains possess one T6SS, and 69 (59.5%) strains have at least two T6SSs (Table S1 in Supporting Information). Using the TssB-based T6SS classification tool provided by SecReT6, most T6SSs are grouped as type i3. At the same time, only four strains have the second T6SSs that belong to the type i2, a kind of T6SS ubiquitous in *Enterobacteriaceae*. With the aid of the T6SS comparison tool, the four extra T6SSs exhibited high sequence similarities to the T6SSs of *K. pneumoniae*, or other species (Figure S3 in Supporting Information), indicating that these different type i2 T6SSs of *S. marcescens* may be horizontally transferred from other species.

We also detected 14,778 non-redundant putative T6SEs, 3,443 T6SIs, 9,422 T6SAs, 65,484 T6SRs and 22,475 recipient susceptibility factors. Notably, 1,521 T6SS-negative genomes still code for the putative T6SEs and T6SIs (Table S2 in Supporting Information). The reason for the presence of such orphan T6SEs and T6SIs in a given bacterial genome needs further investigation. The above examples showed that the *in silico* tools provided by SecReT6 v3 might be helpful for rapid prediction and comparative analysis of T6SSs in bacterial genomes.

Case Study 2: Identification of a novel T6SS regulator and T6SS-dependent killing capacity of *A. baumannii* based on SecReT6 v3

To help examine the T6SS-dependent bacterial killing ability and exploit detection of T6SS-related proteins and mechanisms, we developed a T6SS-dependent killing risk estimation tool and a T6SS-related protein prediction tool. We analyzed T6SSs in the newly completely sequenced genomes of three *A. baumannii* clinical isolates (Table S3 in Supporting Information). Two *A. baumannii* isolates JS-A5 and

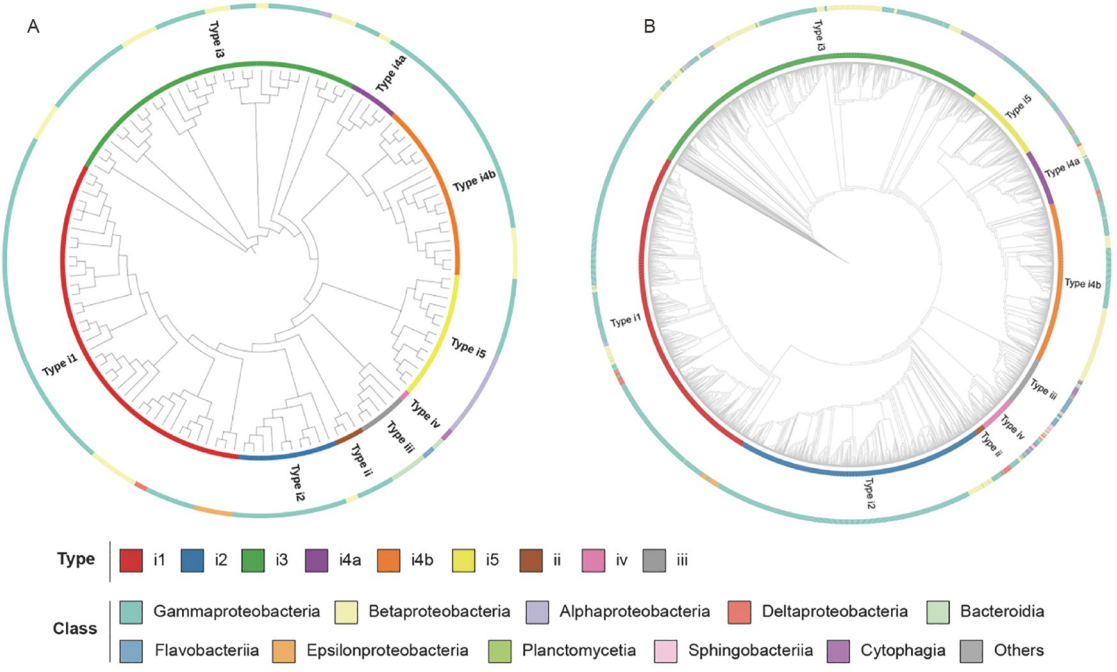

Figure 2 T6SS classification and distribution. A, 211 SeReT6-archived T6SSs with experimental supports. B, 17,212 predicted T6SSs detected in 26,573 bacterial complete genomes available at NCBI. The inner circle shows the known T6SS types, i1, i2, i3, i4a, i4b, i5, ii, iii, and iv. The outer circle shows the distribution of T6SS in different classes. The phylogenetic trees were constructed by FastTree using the multiple sequence alignment of sheath protein TssB generated by MAFFT. The result was visualized by iTOL.

JS-A7 were found to possess one T6SS, while T6SS was not identified in the third isolate JS-A9. We predicted that *A. baumannii* isolates JS-A5 and JS-A7 could kill *E. coli* J53-pF in a T6SS-dependent manner by using the T6SS-dependent killing risk estimation tool, and then conducted gene mutation, complementary assay and *E. coli* killing assay to verify the predictions (Figure 3, Figure S4 in Supporting Information). Compared with the control strain *A. baumannii* ATCC 17978 (Weber et al., 2016), JS-A5 showed a strong T6SS-dependent *E. coli* killing ability consistent with the prediction, while the other T6SS-positive isolate JS-A7 did not. The T6SS-negative isolate JS-A9 did not show *E. coli* killing ability as predicted, possibly due to Insertion Sequences (ISs) (Figure 3), leading to the loss of the T6SS gene cluster.

We speculated that the T6SS of JS-A7 might be repressed due to the negative regulator HvrA encoded by the plasmid pJS-A7. HvrA has an H-NS domain and shares 30.4% BLASTp identities with the SecReT6-archived negative T6SR, VPBB_RS05425 in *Vibrio parahaemolyticus* (Fridman et al., 2020). Thus, we assumed HvrA is the negative T6SR in *A. baumannii*, repressing its T6SS-dependent killing. After deleting the gene *hvrA* according to the previous report (Wang et al., 2019b), JS-A7 showed a strong killing capacity against *E. coli* J53-qF. The subsequent quantitative RT-PCR (qRT-PCR) showed the expression of HvrA can reduce the transcriptional level of *tssD*, *tssB*, and *tssM* (Figure S5 in Supporting Information). Moreover, the expression of HvrA in *A. baumannii* ATCC 17978 and JS-A5 repressed the T6SS-dependent killing ability while the expression HvrA in the ATCC 17978*ΔtssB* and JS-A5*ΔtssB* did not impact their killing abilities (Figure 3). These results suggested that the HvrA is a negative T6SR. In conclusion, with the aid of SecReT6 v3, we detected and verified the T6SS-dependent killing ability of *A. baumannii* clinical isolates and found that the plasmid-coding regulator HvrA inhibited the T6SS-dependent killing *E. coli* ability in *A. baumannii*.

DISCUSSION

A large amount of recent research highlights the importance of T6SS in associated microbial activity. T6SS gets increasing attention for its significant roles in the process, from host interaction to acquiring a metal ion. The research involving T6SS is expanding on numerous grounds, including complex microbial interaction (Basler et al., 2013; Zhao et al., 2018), the evolution of the secretion system (Böck et al., 2017; Bondage et al., 2016), assembly of secretion substrates (Burkinshaw et al., 2018; Quentin et al., 2018), and T6SS regulatory mechanisms (Allsopp et al., 2017; Storey et al.,

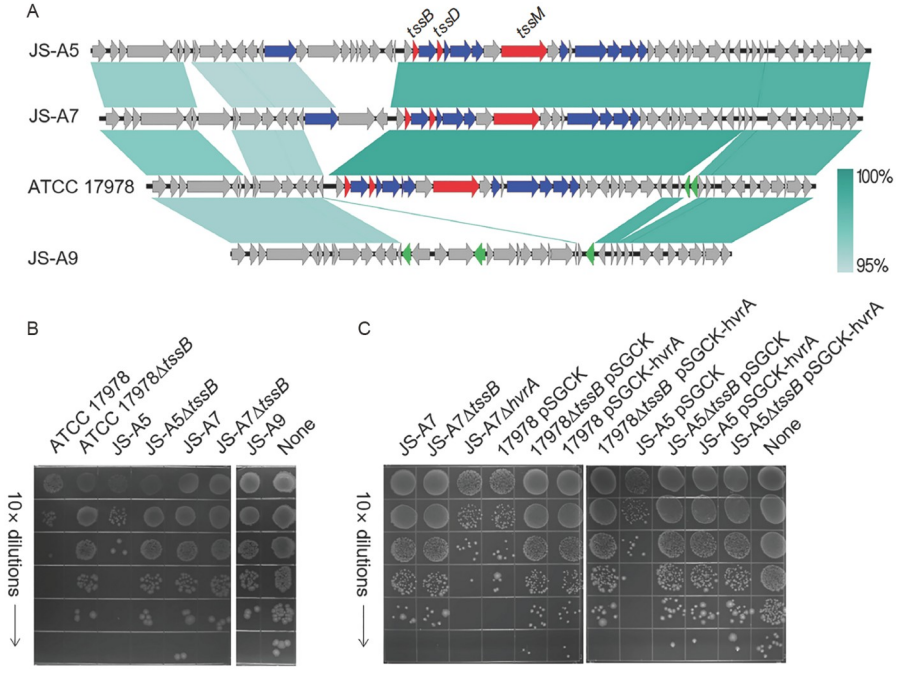

Figure 3 Estimation and verification of the T6SS-dependent killing ability of three *A. baumannii* clinical isolates JS-A5, JS-A7, and JS-A9. A, SeRecT6-detected T6SS gene clusters present in the genomes of JS-A5, JS-A7, ATCC 17978 while absent in JS-A9. The sheath protein gene *tssB*, hemolysin-coregulated protein gene *tssD*, and membrane protein gene *tssM* are highlighted in red and the other T6SS core component genes are marked by blue. IS elements are indicated in green. B, T6SS-dependent killing activities of *A. baumannii* clinical isolates and the *tssB*-deletion mutants against *E. coli* J53-qF. C, The pJS-A7 plasmid-coding protein HvrA negatively regulates T6SS-dependent killing ability in *A. baumannii* strains. The details of the strains and plasmids are described in Table S5 in Supporting Information.

2020). Previous online databases and tools, including the original version of SecReT6 (Li et al., 2015), EffectiveDB (Eichinger et al., 2016) and Bastionhub (Wang et al., 2021) mainly provide the collection of T6SEs and their predictions. With increasing T6SS data, these databases lack the collection of new type T6SEs, like DddA (Jurėnas and Journet, 2021; Mok et al., 2020; Yadav et al., 2021). They cannot match the need for explosively expanding T6SS-associated research fields. The new information on interactions, functions, classifications, and distributions of T6SS and T6SS-related proteins is rapidly increasing, urgently calling for a specific and comprehensive database and tools to support T6SS-associated research.

Here we refactored the T6SS database SecReT6. SecReT6 v3 newly incorporated data of T6SA, T6SR, recipient susceptibility factors with experimental supports which are involved in microbial antagonism, T6SS regulation, and substrate assembly and secretion. The database also incorporated the curated dataset of the known T6SSs, T6SEs, and cognate immunity proteins. To better understand T6SS and T6SS-related proteins and further investigate T6SS-associated molecular mechanisms and regulatory mechanisms, the interactions of these proteins are collected and visualized by interactive charts and diagrams. These data are categorized according to their functions and supplied with detailed information on transmembrane helices, signal peptides, and domains. The new tools for T6SS-related protein prediction, T6SS classification, and T6SS-dependent killing risk estimate together with upgraded T6SS prediction and comparison tools serve all-round and specific support to meet the need in T6SS-associated research.

Based on the database and tools, we have detected 17,212 T6SSs and non-redundant 14,778 T6SEs, 3,443 T6SIs, 9,422 T6SAs, 65,484 T6SRs, and 22,475 recipient susceptibility factors in 26,573 complete bacterial genomes currently available in NCBI. Notably, 7776/26573 (29.3%) Gram-negative bacteria have at least one T6SS, including several clinical pathogens of concern, such as 376/390 (96.4%) *Pseudomonas aeruginosa* strains and 851/916 (92.9%) *Klebsiella pneumoniae* strains. The widespread distribution of T6SS in bacteria indicates the importance of T6SS. All predicted and experimentally validated T6SSs were classified as type i to type iv, based on the sheath protein TssB. The type iv T6SS is newly identified and is closely related to phage with an extracellular contractile injection system (Böck et al., 2017). We additionally found the 17 new T6SS classes in bacteria ranging from *Planctomycetia* to *Negativicutes*. The function and impact of these newly found T6SSs in the T6SS-positive strains should be explored in future microbial research. We further found that most predicted T6SSs in the *S. marcescens* strains belong to type i3, while only four strains contain an extra type i2 T6SS. These additional type i2 T6SSs of *S. marcescens* exhibit high similarity to type i2 T6SS in other species, indicating that *S. marcescens* might have acquired these T6SSs from other species via horizontal gene transfer. In addition, 1,521 T6SS-negative strains still contain T6SEs and T6SIs, so they might have lost T6SS in the evolution or gained orphan T6SEs and T6SIs via horizontal gene transfer. This analysis suggests that SecReT6 may help users to explore new T6SS-related insights.

We further sequenced and analyzed T6SSs in three clinical *A. baumannii* isolates using the tools provided by SecReT6 v3. Combined with verification of *E. coli* killing assays, we showed that *A. baumannii* JS-A5 has a strong T6SS-dependent killing ability to *E. coli* J53-pF, and JS-A7 has a T6SS with potential T6SS-dependent killing ability that maintains silence due to a plasmid-coding negative T6SR HvrA. T6SS-dependent killing risk estimate tool was developed to estimate the T6SS-dependent killing capacity in the predator strain to the prey strain, as T6SS is an extremely essential weapon of Gram-negative bacteria antagonism (Joshi et al., 2017; Zhao et al., 2018). The current version only provides the straightforward estimation of one predator strain to one prey strain, which could not give direct results of the influence of T6SS in mixed bacterial groups.

We believe SecReT6 v3 can facilitate T6SS-associated research like microbial interactions, bacterial life activity, and the investigation of novel T6SS-related proteins.

MATERIALS AND METHODS

Data update by text mining and manual curations

We searched 1,198 articles retrieved from PubMed with the keyword “Type VI Secretion”. After manual curation, we have collected 225 T6SSs, 330 T6SEs, and 156 T6SIs with experimental supports. The T6SSs are classified into nine groups based on the phylogenetic analysis of the sheath proteins TssB (Figure 2). It contains the newly reported T6SS type, T6SS^{iv} (Böck et al., 2017). Moreover, it archived 369 T6SRs, 49 T6SAs, and 62 recipient susceptibility factors with experimental supports (Table S4–S7 in Supporting Information). SecReT6 v3 built the links of these archived proteins to the corresponding experimental literature. All T6SS data are presented with basic information, including locus tag, genome coordinates, and protein ID. Various information on these T6SS-related proteins was incorporated and accessible, such as protein interactions, signal peptides, and transmembrane helices. They are also linked to external public databases, including PDB (https://www.rcsb.org/), KEGG (https://www.kegg.jp/), Uniprot (https://www.uniprot.org/) and Pfam (http://pfam.xfam.org/).

In addition, SecReT6 v3 also contains computationally predicted T6SSs and their associated proteins, including 17,212 T6SSs, 14,778 non-redundant T6SEs, and 3,443 non-

redundant T6SIs detected in 26,573 complete bacterial genomes currently available in NCBI (Table S1 in Supporting Information). They can be downloaded from SecReT6 v3.

Convenient interface and flexible exhibition

We have refactored the database and newly added various aspects of experimentally validated T6SS data, with detailed information shown by a convenient and user-friendly interface of T6SS map in the organism, T6SS cluster visualization, and the interaction of T6SS and T6SS-related proteins. SecReT6 v3 provides various modules, such as "Browse", "Statistics", "Tools", "Download", and "References". Data of T6SS and T6SS-related proteins were exhibited via several flexible web interfaces in the "Browse" module, divided into six portions, including T6SS, T6SE, T6SI, Recipient susceptibility factor, T6SA, and T6SR according to the content type. For convenient use, all data were further exhibited via "Taxonomy", "Replicon", and "Classification" according to function or protein phylogenetic tree in the "Statistics" module. All data in each portion were further divided into several classes according to the type of T6SS data. The "Tools" portion provides easy-to-use tools to predict and analyze T6SS, including T6SS prediction, T6SS classification, T6SS-related protein prediction, T6SS-dependent killing risk estimation, and T6SS comparison.

Integration of T6SS prediction and comparison tools

SecReT6 v3 provides five newly developed or updated online tools for investigating the putative T6SSs and their related proteins, including (i) T6SS-related protein prediction tool, (ii) T6SS classification tool, (iii) T6SS comparison, (iv) T6SS-dependent killing risk estimation, (v) T6SS prediction tool. The details about these tools are available in (Table S4–S7 in Supporting Information).

The T6SS-related protein prediction tool (Figure S6 in Supporting Information) was constructed to detect the putative T6SS-related proteins, ranging from the conserved T6SS structure component proteins to the various T6SEs, T6SIs, T6SRs, T6SAs, and recipient susceptibility factors, by using HMMER (27) and NCBI BLASTp (Camacho et al., 2009). Then, the multiple sequence alignment was generated by Clustal Omega (Sievers and Higgins, 2018) and was visualized with R package msa (Bodenhofer et al., 2015). The signal peptide and transmembrane region are predicted by SignalP (Almagro Armenteros et al., 2019) and TMHMM (Krogh et al., 2001), respectively.

T6SS classification tool (Figure S7 in Supporting Information) was designed based on the phylogenetic relationship of the sheath protein TssB/IgIA/gp18/VipA of the known T6SSs. The query TssB protein sequence (the user input) is added in the MAFFT (Katoh and Standley, 2013) pre-constructed alignment of all experimentally confirmed TssB proteins. The output phylogenetic tree is constructed by FastTree (Price et al., 2010) and visualized by ggtree (Yu et al., 2018).

T6SS-dependent killing risk estimation tool (Figure S8 in Supporting Information) employs BLASTp (Camacho et al., 2009) (or hmmsearch (Finn et al., 2011)) to detect T6SS and T6SE in the predator strain genome, as well as T6SI and recipient susceptibility factor in the prey strain genome to evaluate the killing capacity of the predator strain. Only when the predator strain lacks the T6SS can it be assessed that the prey has no risk of being killed in a T6SS-dependent manner. When the predator contains T6SS and antibacterial T6SEs, while the prey contains the T6SIs that could be immune to all T6SEs in the predator, it can be assessed that the prey has a low risk of being killed. When the prey lacks T6SIs to immune T6SEs in the predator with a functional T6SS, it has a high risk of being killed. The recipient susceptibility factor, the non-specific immunity regulator or mechanism that responds to an exogenous T6SS attack, is also detected to support the exploration of the T6SS-associated bacterial killing.

The upgraded T6SS prediction tool (Figure S9 in Supporting Information) incorporated T6SS classification, T6SS-related protein prediction, alignment function and T6SS cluster prediction. Auto-selection of T6SSs is achieved by MultiGeneBLAST (Medema et al., 2013) and then T6SS gene clusters would be aligned by BLASTn and visualized by Easyfig (Sullivan et al., 2011).

Strains, plasmids, and culture conditions

All strains and plasmids used are provided in Table S3 in Supporting Information, and culture conditions were supplied on the Supplemental Methods in Supporting Information.

E. coli killing assay

The killing assay was performed as previously described (Weber et al., 2016) with minor modifications described in supplemental methods.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

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