The *Vibrio* Type III Secretion System 2 is not restricted to the *Vibrionaceae* and encodes differentially distributed repertoires of effector proteins

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ABSTRACT

Vibrio parahaemolyticus is the leading cause of seafood-borne gastroenteritis worldwide. A distinctive feature of the O3:K6 pandemic clone, and its derivatives, is the presence of a second, phylogenetically distinct, Type III Secretion System (T3SS2) encoded within the genomic island VPaI-7. The T3SS2 allows the delivery of effector proteins directly into the cytosol of infected eukaryotic cells to subvert key host cell processes, critical for *V. parahaemolyticus* to colonize and cause disease. Furthermore, the T3SS2 also increases the environmental fitness of *V. parahaemolyticus* in its interaction with bacterivorous protists; hence it has been proposed that it contributed to the global oceanic spread of the pandemic clone. Several reports have identified T3SS2-related genes in *Vibrio* and non-*Vibrio* species, suggesting that the T3SS2 gene cluster is not restricted to the *Vibrionaceae* and can mobilize through horizontal gene transfer events. In this work, we performed a large-scale genomic analysis to determine the phylogenetic distribution of the T3SS2 gene cluster and its repertoire of effector proteins. We identified putative T3SS2 gene clusters in 1130 bacterial genomes from eight bacterial genera and five bacterial families. A hierarchical clustering analysis allowed us to define six T3SS2 subgroups (I-VI) with different repertoires of T3SS2 effector proteins, redefining the concepts of T3SS2 core and accessory effector proteins. Finally, we identified a subset of T3SS2 gene clusters that lack most T3SS2 effector proteins described to date and provided a list of 10 novel effector candidates for this subgroup through bioinformatic analysis. Collectively, our findings indicate that the T3SS2 extends beyond the *Vibrionaceae* family and suggest that different effector protein repertories could have a differential impact on the pathogenic potential and environmental fitness of all the bacteria that have acquired the *Vibrio* T3SS2 gene cluster.

DATA SUMMARY

1. All genome sequences used in this study were downloaded from the National Center for Biotechnology Information (NCBI) RefSeq or GenBank databases (See **Table S1** for accession numbers).

2. Files for the T3SS2 reconstructed phylogenetic tree (Newick tree and MSA fasta file), hierarchical clustering data analysis file from MORPHEUS, **Table S1** with genome accession numbers and all the data of the absence/presence of T3SS2-related components, and **Table S2** with the prediction of novel effector proteins are available as part of the online Supporting Dataset at the Zenodo data repository (https://doi.org/10.5281/zenodo.7016552). The T3SS2 phylogenetic tree can be interactively visualized in https://itol.embl.de/tree/1901619012537471162695906 7#

INTRODUCTION

Vibrio parahaemolyticus is a Gram-negative bacterium that resides in marine and estuarine environments [1, 2]. In 1996, a pandemic clone of the O3:K6 serotype emerged and spread around the globe, becoming the leading cause of seafood-borne gastroenteritis worldwide [3]. Whole-genome sequencing of the pandemic strain RIMD2210633 revealed the presence of a novel Type III Secretion System (T3SS2) encoded within an 80-kb genomic island on chromosome 2, known as the *V. parahaemolyticus* pathogenicity island 7 (VPaI-7) [4]. While all *V. parahaemolyticus* strains encode a T3SS on chromosome 1 (T3SS1), only *V. parahaemolyticus* strains derived from the original pandemic clone harbor the phylogenetically distinct T3SS2 [4].

T3SS are complex nanomachines that enable Gram-negative bacteria to deliver proteins, known as effectors, directly from the bacterial cytosol into the cytosol of eukaryotic cells [5, 6]. The translocation of effectors into target cells enables bacteria to subvert a wide variety of host cell functions [7–9]. In addition, the specific repertoire of proteins delivered to infected cells determines which cellular networks are subverted by the bacterium and ultimately influence the outcome of the infection [5, 6, 10]. In this context, even closely related T3SSs can have different repertoires of effector proteins due to the due to horizontal gene transfer (HGT) and/or genomic rearrangement events [11, 12]. A recent global survey described that T3SS are widely distributed among Gram-negative bacteria, identifying 174 nonredundant T3SS from 109 distinct bacterial genera [13]. T3SS have been classified in 13 (I-XIII) major categories based on sequence divergence of T3SS core components [13]. These T3SS have been classified in 13 (I-XIII) major categories based on sequence divergence of T3SS core components [13]. According to this study, the *Vibrio* T3SS1 is part of category Ib, and the T3SS2 is the founding member of the T3SS phylogenetic category X [13].

Currently, the T3SS2 is considered the main virulence factor of *V. parahaemolyticus*. Studies in animal models of infection revealed that, while the T3SS1 plays a minor role during infection, the T3SS2 is essential for the enterotoxicity and the development of the clinical signs of the disease [14–17]. Therefore, the T3SS2 is considered the major virulence factor of pandemic *V. parahaemolyticus* [18]. The gene expression of the T3SS2 gene cluster is induced by bile salts [19, 20] through a regulatory network involving the VtrA, VtrC, and VtrB proteins [19, 21, 22] and the T3SS2 function requires host cell-surface fucosylation for efficient delivery of effector protein into infected cells [23]. Notably, the importance of the T3SS2 goes beyond increasing the pathogenic potential of pandemic strains, as the T3SS2 increases the environmental fitness of *V. parahaemolyticus* by promoting bacterial survival against predatory amoeba [24].

Phylogenetic analysis based on the amino acid sequence divergence of T3SS structural components has identified two distinct lineages of the T3SS2, known as phylotypes T3SS2α and T3SS2β [25, 26]. T3SS2α and T3SS2β gene clusters share a similar genetic organization and a subset of effector proteins (**Fig. 1**) [18]. A more recent study has proposed an additional category within T3SS2β, known as T3SS2γ [27]. The T3SS2γ is very similar to the T3SS2β gene cluster but has some gene content differences, such as the presence of a *tdh* gene inserted within the T3SS gene cluster [27] (**Fig. 1**).

Most of our knowledge of the T3SS2 comes from studies of the T3SS2α of *V. parahaemolyticus* strain RIMD2210633 and T3SS2α *V. cholerae* strain AM-19226 (reviewed in [18, 28]). In this context, 15 different T3SS2 effector proteins have been identified, of which only 5 are shared between *V. parahaemolyticus* RIMD2210633 and *V. cholerae* AM-19226 [29–42]. These 5 effectors are therefore called core effector proteins, while the remaining 10 are known as accessory effector proteins [18]. While useful, this classification has the limitation of comparing the distribution of effector of only 2 *Vibrio* strains of the same T3SS2 phylotype.

The T3SS2 gene cluster is not restricted to *V. parahaemolyticus* as similar T3SSs have been found in other species such as non-O1/non-O139 *V. cholerae*, *V. mimicus* and *V. anguillarum* [25, 43–46]. Furthermore, several reports have identified the presence of one or more T3SS2-related genes in strains of bacterial genera other than *Vibrio*, including *Aeromonas* [47], *Grimontia* [20], *Photorhabdus* [48, 49], *Providencia* [50] and *Shewanella* [49, 51]. Altogether, these reports suggest that the presence of T3SS2 gene clusters extends beyond the *Vibrionaceae* family and could have been acquired through HGT events. Nevertheless, these correspond to isolated observations performed in a limited number of strains and bacterial genomes. Therefore, the overall taxonomic and phylogenetic distribution of the T3SS2 and its effector proteins in bacterial genomes is currently unknown.

To address this issue, in this study, we performed a large-scale bioinformatic analysis to gain insight into the phylogenetic distribution of the T3SS2 gene clusters, including effector proteins. Our analysis identified 1030 bacterial genomes encoding putative T3SS2 gene clusters distributed over 43 different bacterial species from 8 bacterial genera. In addition, we classified these T3SS2s into 6 different subgroups in terms of the repertoire of effector proteins they possess. Our work provides evidence that the T3SS2 extends beyond the *Vibrionaceae* family and that acquisition of both the T3SS2 and different repertoires of effector proteins could have an impact on the pathogenic potential and environmental fitness of these bacteria.

Impact Statement

One of the distinctive features of the O3:K6 pandemic clone of *Vibrio parahaemolyticus* is the presence of a phylogenetically distinct Type III Secretion System (T3SS) known as T3SS2. T3SS are complex nanomachines that allow the delivery of effector proteins directly into the cytosol of eukaryotic cells to manipulate different cellular functions. The *Vibrio* T3SS2 is the major virulence factor of the pandemic clone and a pivotal contributor to the pathogenesis and environmental fitness of the bacterium. Several studies have identified the T3SS2 gene cluster in other bacterial species beyond the *Vibrionaceae* family, suggesting that this T3SS can be mobilized through horizontal gene transfer events. In this work, we performed a phylogenetic distribution analysis of the T3SS2 gene cluster in publicly available bacterial genome sequences. We identified putative T3SS2 gene clusters in 1130 bacterial genomes from 43 different bacterial species from 8 genera and 5 bacterial families. Furthermore, we classified these T3SS2s into 6 different subgroups (I-VI) in terms of their repertoire of effector proteins. Our findings indicate that the T3SS2 is not restricted to the *Vibrionaceae* family and suggest that different effector protein repertories could have a differential impact on the pathogenic potential and environmental fitness of all the identified bacteria that have acquired this T3SS2.

METHODS

In silico identification and analyzes of T3SS2 loci and components

To identify putative T3SS2 loci, the amino acid sequence of the VPA1338 (SctN) and VPA1339 (SctC) proteins of *V. parahaemolyticus* RIMD 2210633 were used as queries in BLASTp, to perform analyses [52] using publicly available bacterial genome sequences of the National Center for Biotechnology Information (NCBI) database (February 2021). A cut-off of 70% identity and 80% sequence coverage threshold were used to select for positive matches. All genome sequence assemblies with positive hits were downloaded from the NCBI RefSeq or GenBank databases (See **Table S1** for accession numbers and metadata) and used to build local BLAST databases. To identify the remaining 37 T3SS2-related components in these 1130 genomes, the nucleotide and aminoacid sequence of each component (**Table S2**) was used as queries for either tBLASTx or BLASTp analyzes, respectively. A 50% identity and 80% sequence coverage threshold were used to select for positive matches. For comparative genomic analysis, nucleotide sequences were visualized and analyzed by the Artemis version 18.1 [53], the Artemis Comparison Tool (ACT) release 6 [54], the multiple aligner Mauve [55] and EasyFig v2.2.2 [56]. For the hierarchical clustering analysis, a presence/absence matrix of each T3SS2 component was constructed for each bacterial genome (**Table S1**) and uploaded as a *csv* file to the online server of the Versatile matrix visualization and analysis software MORPHEUS (one minus Pearson correlation, average linkage method) (https://software.broadinstitute.org/morpheus). The hierarchical clustering data analysis file from MORPHEUS is available as part of the online Supporting Data at the Zenodo data repository (https://doi.org/10.5281/zenodo.7016552). The presence and absence of each identified component were visualized as a colored matrix generated in GraphPad Prism version 9 (GraphPad Software, San Diego, California, USA).

T3SS2 phylogenetic analyses

The 1130 concatenated SctN and SctC amino acid sequences were aligned with ClustalW using the Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0 [57]. The phylogenetic tree was built from the alignments obtained from MEGA by performing a bootstrap test of phylogeny (1000 replications) using the Maximum-Likelihood (ML) method with a Jones-Taylor-Thornton (JTT) + CAT correction model using FastTree2 (Galaxy Version 2.1.10+galaxy1) [58] in the Galaxy platform (https://usegalaxy.org/) [59]. The phylogenetic tree was further visualized and edited to include important metadata with iTOL [60]. Files for the T3SS2 reconstructed phylogenetic tree (Newick tree) is available as part of the online Supporting Data at the Zenodo data repository (https://doi.org/10.5281/zenodo.7016552). The T3SS2 phylogenetic tree can be interactively visualized in https://itol.embl.de/tree/1901619012537471162695906 7#

Identification of novel T3SS2 effector candidates

Each Open Reading Frame (ORF) within the predicted T3SS2 gene clusters of Subgroup VI (including 1 kb upstream and downstream of the first and last predicted component, respectively) was analyzed by means of the EffectiveDB [61], Bastion3 [62] and BEAN2.0 [63] pipelines to identify potential T3S signal sequences. ORFs with positive hits in at least one of these pipelines was further analyzed to predict putative functional domains with the InterProScan [64] and NCBI-CDD [65]. Finally, each ORF was also analyzed by the we used the structure-based homology tool HHpred [66]. Multiple sequence alignments were performed by MAFFT [67] and T-Coffee Expresso [68] and visualized by ESPript 3.0 [69].

RESULTS AND DISCUSSION

T3SS2 gene clusters extend beyond the *Vibrionaceae* family

We screened all publicly available bacterial genomes in the NCBI database to identify the full breadth of bacterial species harboring putative T3SS2 gene clusters. First, we performed BLASTP analysis in search for homologs of the VPA1338 (SctN) and VPA1339 (SctC) proteins of *V. parahaemolyticus* RIMD 2210633. SctN and SctC were chosen as they are two of the most conserved T3SS structural proteins and are often used for phylogenetic clustering and classification of T3SSs [13, 70]. We used stringent cut-offs to avoid identifying homolog proteins from phylogenetically close T3SSs (≥70 % of identity and over ≥80 % of coverage). Our analysis identified the presence of VPA1338 and VPA1339 homologs in 1130 bacterial genomes (**Fig. 2, Table S1**).

To determine if these 1130 bacterial genomes could encode complete T3SS2 gene clusters, we further screened each genome for the presence of the 37 T3SS2-related components previously identified in *V. parahaemolyticus* RIMD 2210633 (T3SS2α), *V. parahaemolyticus* MAVP-R (T3SS2β) and *V. cholerae* AM-19226 T3SS2α (**Table S1**). These include ORFs encoding 16 structural proteins, 15 bacterial effector proteins, 3 regulatory proteins (VtrA, VtrB, and VtrC), and each of the 3 hemolysins linked to T3SS2 gene clusters (TDH_{Vp}, TRH_{Vc} and TRH_{Vp}). Most of the 1130 bacterial genomes (1106) encoded each of the T3SS structural genes suggesting that they most likely harbor complete T3SS2 gene clusters (**Fig. 3**). The exception was 24 *Vibrio anguilarum* genomes that only possess 8 of the 16 T3SS2 structural components (**Table S1**). This was not unexpected as it has been previously reported that some *V. anguillarum* strains lack most of the T3SS2 structural components due to a large internal deletion of the T3SS2 gene cluster [71], suggesting that these strains do not encode functional T3SS2s.

Taxonomic distribution analysis identified putative T3SS2 gene clusters in 43

bacterial species from 8 bacterial genera (*Vibrio*, *Photobacterium*, *Grimontia*, *Photorhabdus*, *Yersinia*, *Shewanella,* and *Aeromonas*) and 5 distinct families (*Aeromonadaceae*, *Morganellaea*, *Shewanellaceae*, *Vibrionaceae,* and *Yersiniaceae*) (**Fig. 2A**). In some genus, we identified multiple species harboring putative T3SS2 gene clusters while in other genus we could identify as little as one bacterial species harboring T3SS2 gene components (**Fig. 2A**). In terms of bacterial species, the genus *Vibrio* contained the largest number of different species harboring putative T3SS2 gene clusters (29 species), followed by the *Photorhabdus*, *Shewanella* and *Providencia* (each with 3 species), the *Yersinia* genus (2 species) and the *Photobacterium*, *Grimontia,* and *Aeromonas* each with a single species. Finally, our analysis did not identify bacterial strains harboring multiple T3SS2 gene clusters within their genomes.

Interestingly, most of the different bacterial species identified to harbor putative T3SS2 gene clusters can be found in aquatic habitats, suggesting that acquisition and transfer of the T3SS2 actively occur in this environment. Among the exceptions was the identification of T3SS2 gene clusters in genomes of strains of the *Photorhabdus* genus (**Fig. 2A** and **Table S1**). Bacteria from the genus *Photorhabdus* comprise different bacterial species that are important insect pathogens, including some that can also cause human disease [72]. Wilkinson et al.[48], previously identified a T3SS2 gene cluster in the genome of *Photorhabdus asymbiotica* strain ATCC43949. Our analysis expanded this finding by identifying complete T3SS2 gene clusters in different strains of *P. australis*, *P. heterorhabitis* and *P. asymbiotica* species (**Fig. S2** and **Table S1**). Every T3SS2 gene cluster identified is inserted in the exact genetic location compared to the *P. laumondii* strain TT01 genome, as previously reported for *P. asymbiotica* strain ATCC43949 [48]. We also identified T3SS2 gene clusters in 1 strain of *Yersinia kristensenii* and 2 strains of *Yersinia enterocolitica* (**Fig. 2A** and **Table S1**). Interestingly, while *Yersinia enterocolitica* can be found in aquatic environments, the 2 strains with putative T3SS2 gene clusters

were isolated from wild ungulate carcasses and belong to the highly virulent biotype 1B [73].

Altogether, the taxonomic distribution of T3SS2 gene clusters in bacterial genomes indicates that this secretion system is not restricted to the *Vibrionaceae* family and supports the notion that T3SS2 gene clusters can mobilize through horizontal gene transfer events among environmental bacteria.

The T3SS2β phylotype is most abundant and divergent than the phylotype T3SS2α

The T3SS2 is divided into two major phylotypes known as T3SS2α and T3SS2β [18], and an additional subcategory within T3SS2β known as T3SS2γ [27]. To determine the distribution of T3SS2α, T3SS2β and T3SS2γ, among the 1130 T3SS2s identified, we performed a phylogenetic analysis using concatenated SctN and SctC aminoacidic sequences. We also included the concatenated SctN and SctC sequences of the *Agrobacterium radiobacter* K84 and *Rhizobium etli* CIAT 652 T3SSs, which are representatives of T3SS category XI, the closest phylogenetic category to the *Vibrio* T3SS2 [13]. The phylogenetic clustering analysis showed that all the 1030 putative T3SS2s grouped closely together while the T3SS of *Agrobacterium radiobacter* K84 and *Rhizobium etli* CIAT 652 cluster in a more distant branch (**Fig. 2B**), supporting the notion that all the 1130 T3SS identified in this work correspond to bona fide T3SS2s. Our analysis also revealed that 36,6% (n= 414) of the identified T3SS2s belonged to phylotype T3SS2α and 61% (n= 689) to phylotype T3SS2β. The phylogenetic analysis also revealed that the greatest sequence divergence was observed within the T3SS2β phylotype (**Fig. 2C**).

As mentioned above, recent reports have created a subcategory within T3SS2β of *V. parahaemolyticus* known as T3SS2γ (based mainly on the presence of both the *tdh* and

trh genes) [27]. To determine the percentage of T3SS2γ gene clusters within the T3SS2β family, we performed a gene content analysis based on the definition of T3SS2γ gene clusters proposed by Xu et al., [13]. The analysis identified that 40,5% (n= 280) of T3SS2β gene clusters could be classified as T3SS2γ. Notably, the presence of T3SS2γ did not cluster to any major clade of the T3SS2 phylogenetic tree (**Fig. 2B**), supporting the notion that T3SS2γ is not a third T3SS2 phylotype but rather a subcategory within T3SS2β.

Taxonomic distribution analysis identified the presence of T3SS2α and T3SS2β in strains of each of the 5 bacterial families (**Fig. 2A and 2B**), with no clear overrepresentation of any phylotype within a particular bacterial family. Nevertheless, T3SS2β was present in a more significant number of different bacterial species (37 out of 44) in comparison to T3SS2α (19 out of 44 species) (**Fig. 2A**). Finally, the T3SS2γ subcategory was identified solely in *V. parahaemolyticus* strains which agree with previous reports [27]. Since many *V. parahaemolyticus* strains which harbor T3SS2 gene clusters are related to the RIMD 2210633 pandemic clone, we analyzed the distribution of T3SS2 phylotypes among different *V. parahaemolyticus* Sequence Types (STs). Our analysis showed that T3SS2α was overrepresented in *V. parahaemolyticus* strains from ST3 (44%), ST120 (11,5%), ST189 (7%) and ST332 (16,4%) while the T3SS2β was overrepresented in strains from ST3 (11,5%), ST36 (25,2%) and ST631 (7,5%) (**Fig. S1**).

T3SS2 gene clusters share a high degree of synteny

To gain insight into the genomic context of the identified T3SS2 gene clusters, we performed a comparative genomic analysis in a subset of bacterial strains with complete or almost complete bacterial genomes (large enough contigs to allow a comparative analysis). Notably, there was a high gene cluster synteny between the T3SS2 gene of *V. parahaemolyticus* RIMD2210633 and the gene clusters of bacterial species of different

genera (e.g., *Providencia, Photobacterium, Grimontia* and *Photorhabdus*) (**Fig. 3 and Fig. S2**). A high gene cluster synteny was also observed between the T3SS2 gene clusters with a lower sequence identity, such as the gene clusters of *Aeromonas* sp. DNP9 and *Yersinia enterocolitica* Y1Wildboar1B (**Fig. 3C**).

In terms of genome location, in addition to the described chromosomal location of T3SS2 within VPAI-7 of *V. parahamolyticus* RIMD2210633 [4], we identified a T3SS2 gene cluster within the plasmid of a recently sequenced *V. meditteraniae* strain 117-T6 (CP033579) [74] (**Fig. 4A**). Another interesting observation was the presence of a T3SS2 gene cluster inserted right next to a T3SS1 gene cluster in *V. campbelli* strain UMTGB204. In this strain both T3SSs are inserted within the vicinity of the same serine tRNA (**Fig. 4B**), in which the T3SS1 of *V. parahaemolyticus* RIMD 2210633 is located. Finally, in each of the T3SS2 gene clusters identified in *Grimontia hollisae* strains, a large gene encoding a putative adhesin (SiiE-like adhesin) is encoded in the middle of the T3SS2 gene cluster (**Fig. 4A**).

Hierarchical clustering analysis identifies 6 subgroups with different repertoires of effector proteins

While phylogenetic analysis of T3SSs using core structural components provides insight into the evolution of T3SSs, the acquisition, transfer, or loss of accessory and effector encoding genes provides an additional layer of complexity to the evolution of these systems [11, 13, 70]. To gain further insight into this diversity, we performed a hierarchical clustering analysis of 37 T3SS2-related (**Fig. 5**). For the analysis of the effector proteins, we included each T3SS2 effector described for either *V. parahaemolyticus* RIMD 2210633 or *V. cholerae* AM 19226 [18]. The hierarchical clustering analysis allowed us to identify 6 major subgroups (I to VI) with similar gene content profiles (**Fig 5**, **Table S1**).

Most structural components were highly conserved in all subgroups, except for the

hydrophilic translocator VopW of subgroups I and VI and the VopB2 and VopD2 translocon proteins of subgroup VI. The *vtrA*, *vtrB,* and *vtrC* genes, which encode the regulatory complex that induces the expression of T3SS2 gene clusters in response to bile [19, 22], were also highly conserved, except for subgroups III and VI, where many bacterial strains harbored divergent homologs of VtrA and VtrC (**Fig. 5**).

Since most structural genes were highly conserved across all genomes, the hierarchical clustering analysis mainly classified each T3SS2 regarding the repertoire of effector proteins identified. As shown in **Fig. 6A**, each T3SS2 subgroup was enriched in a distinct set of effector proteins and associated hemolysins. While most subgroups were enriched for a combination of 10-12 different T3SS2 effectors (subgroups I to V), subgroup VI was vastly underrepresented in terms of the presence of known effector proteins. This subgroup was primarily enriched in the VgpA effector protein, lacking most other known T3SS2 effector proteins. The presence of VgpA homologs in each subgroup supports this effector´s critical role in T3SS2 function [39, 75]. As expected, the current phylotype classification (T3SS2α versus T3SS2β) was a poor predictor of the presence/absence of individual effector proteins (**Fig. 6A and B**), highlighting the utility of a gene-content classification scheme.

Interestingly, most T3SS2 subgroups were enriched for genomes of particular bacterial species (**Fig. 6B**): i) Subgroup I included strains from 4 different bacterial species (*P. damselae*, *V. cholerae*, *V. mimicus,* and *Vibrio sp*.); ii) Subgroup II included strains only from *Grimontia hollisae* species; iii) Subgroup III included *V. parahaemolyticus* strains exclusively; iv) Subgroup IV included strains from 3 species (*V. cholerae*, *V. mimicus* and *V. parahaemolyticus*); v) Subgroup V included strains from 5 species (*V. parahaemolyticus*, *V. cholerae*, *V. diabolicus*, *V. mimicus* and *Vibrio sp*.); and subgroup VI harbored the most diverse set of bacterial species with a total of 39 bacterial species (**Fig. 6B**). Subgroup VI was also the only subgroup with bacterial strains beyond the

Vibrionaceae family. Finally, while no T3SS2 subgroup was exclusive to a particular T3SS2 phylotype some subgroups were indeed overrepresented by different phylotypes. Subgroups I, III and IV were overrepresented in strains from the T3SS2α phylotype, while subgroups II, V and VI were overrepresented in strains from the T3SS2β phylotype. Finally, every *V. parahaemolyticus* strain of the T3SS2γ subcategory clustered within subgroup V (**Table S1**).

Altogether, the hierarchical clustering analysis suggests that T3SS2s have acquired a diverse set of effector proteins which could ultimately impact on the contribution of each T3SS2 to the pathogenic potential and environmental fitness of different bacterial strains.

T3SS2 subgroup VI encodes 10 novel effector candidates

As mentioned above, the T3SS2 gene clusters of subgroup VI stand out as they are the most taxonomically diverse (**Fig. 6B**) and lack most of the known T3SS2 effector proteins (**Fig. 5, 6A**). The high degree of synteny of these gene clusters to the T3SS2 gene cluster of *V. parahaemolyticus* RIMD2210633 (**Fig. 3 and Fig. S2**) and their distribution in the T3SS2 phylogenetic tree (**Fig 2**) suggests that these are not part of a phylogenetically distinct T3SS2, but rather have a different set of effector proteins. To test this hypothesis, we extracted the T3SS2 gene cluster from selected bacterial genomes of Subgroup VI (strains with closed genomes or sufficiently large contigs to include complete T3SS2 gene clusters) and performed bioinformatic analysis to identify putative T3SS2 effector candidates. We analyzed each ORF of these T3SS2 clusters based on 4 criteria, including: i) identification of putative T3SS secretion signals by T3SS effector prediction pipelines (BEAN2.0, Bastion3 and EffectiveDB); ii) bioinformatic analysis to identify conserved functional domains (InterProScan, NCBI-CDD); iii) sequence identity to known T3SS effector proteins of other bacterial species (BLAST); and iv) functional predictions

via remote homology searches using the HHpred pipeline.

Our analysis identified a set of 10 novel effector candidates that harbored putative T3SS secretion signal sequences and putative functional domains present in known T3SS effector proteins. These included putative ADP-ribosyltransferases, tyrosine phosphatases, glycosyltransferases and Rho-activating proteins (**Table 1 and Fig. 7A**). In addition, 5 of these ORFs encode homologs of known T3SS effector proteins or toxins such as the effector proteins OspC1, AoPH and VopC of *S. flexneri*, *A. hydrophila* and *V. parahaemolyticus* respectively (**Table 1 and Fig. 7A**).

Interestingly, one candidate harbored a predicted GDSL lipase domain (IPR001087) and a 22,4% sequence identity to the VPA0226 protein of *V. parahaemolyticus* (**Fig. S4**). VPA0226 is a secreted lipase required for *V. parahaemolyticus* RIMD2210633 to egress from the infected cell. Notably, in *V. parahaemolyticus* VPA0226 is not secreted by the T3SS2 but rather by the T2SS [76]. The 10 effector candidates identified were present in diverse bacterial species of the T3SS2 subgroup VI, except for the putative tyrosine phosphatases (DYE18_RS08865) that also was identified in *Grimontia hollisae* strains of T3SS2 subgroup II (**Fig. 7B and Fig. 7C**).

Overall, our analysis suggests that subgroup VI encodes a unique set of novel T3SS2 effector proteins not present in any other T3SS2 subgroup. Further work is needed to confirm that each candidate corresponds to T3SS2 effector proteins.

Revisiting the current classification of core and accessory T3SS2 effector proteins

Recently, Hu et al.[13], proposed a classification of T3SS2 effector proteins based on the presence of these effectors in *V. parahaemolyticus* and *V. cholerae.* Effectors present in both *V. cholerae* and *V. parahaemolyticus* are known as core effectors, and those present in either *V. cholerae* or *V. parahaemolyticus* have been called accessory

effector proteins. While useful, this classification relies on analyzing only two strains of two bacterial species.

To revisit this classification, we analyzed the presence/absence of each T3SS2 effector protein in the 43 bacterial species in which we have identified putative T3SS2 gene clusters. As shown in **Fig. S3**, only VgpA was present in at least one strain of most bacterial species (88%). The high prevalence of this effector protein could be explained by its additional structural role [39]. Another effector, VopT, was identified exclusively in *V. parahaemolyticus* strains. The remaining 13 T3SS2 effector proteins were present at various degrees with no overrepresentation in any bacterial species. Altogether, our data show a wide distribution of T3SS2 effector proteins in different bacterial species and that there is no clear correlation between the number of bacterial species that harbor a particular effector protein, which is the basis of the current classification scheme. In this context, we propose that a better alternative is to classify T3SS2s based on the repertoire of their effector proteins in the context of all identified T3SS2 loci in bacterial genomes (subgroups I-VI). One of the limitations of this classification scheme is that it is dynamic, as it may change over time if novel effector encoding genes are acquired through HGT or if some are lost due to genomic rearrangements.

CONCLUSION

The acquisition and transfer of T3SS gene clusters can potentially increase the virulence and the environmental fitness of the recipient bacteria. In this context, it has been proposed that the acquisition of the T3SS2 gene cluster by *V. parahaemolyticus* was a crucial factor in the global spread and increased pathogenic potential of the O3:K6 pandemic clone and its derivatives. In this study, we performed a genome-wide analysis to determine the phylogenetic distribution of the *Vibrio* T3SS2 gene cluster in public genome databases. Our analysis revealed that T3SS2 gene clusters extend beyond the *Vibrionaceae* family, share a high degree of synteny and that it is possible to categorize T3SS2 in six different subgroups (I-VI) in terms of their repertoire of effector proteins. Notably, it is plausible to think that acquisition of the T3SS2 gene cluster by HGT events in each of the bacteria identified in this study could increase their human pathogenic potential and environmental fitness, just as it has been proposed for the *V. parahaemolyticus* pandemic clone. Finally, since the repertoire of T3SS effector proteins delivered to the target cells ultimately defines the cellular outcome of an infection, we believe that future studies should focus on testing the contribution of each repertoire of T3SS2 effector proteins to the pathogenic potential and environmental fitness of these bacterial species.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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AUTHOR CONTRIBUTIONS

Sebastian Jerez: Investigation, Visualization, review and editing final manuscript.

Nicolas Plaza: Investigation, Visualization, review and editing final manuscript.

Italo M Urrutia: Investigation, Visualization, review and editing final manuscript.

Veronica Bravo: Investigation, Visualization, review and editing final manuscript.

Carlos J Blondel: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Resources, Data curation, Writing-Original Draft and review and editing., Funding acquisition, Supervision and Project administration.

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FIGURE LEGENDS

Fig. 1. The T3SS2 is divided into two major phylotypes T3SS2α and T3SS2β, and a subcategory within T3SS2β known as T3SS2γ. Schematic depiction of a comparison of the T3SS2 gene clusters in *V. cholerae* AM-19226, *V. parahaemolyticus* RIMD2210633, *V. parahaemolyticus* MAVP-R, and MAVP-Q. BLASTn alignment was performed and visualized using Easyfig. Genes encoding different components are highlighted in color.

Fig. 2. The T3SS2 extends beyond the *Vibrionaceae* family. (A) Identification and taxonomic distribution of T3SS2 gene clusters in bacterial genomes. The presence of T3SS2 gene clusters from phylotypes T3SS2α (red) and T3SS2β (blue) are shown for each taxonomic category. The numbers correspond to the total numbers of bacterial genomes within each taxonomic category that harbored a putative T3SS2 gene cluster. (B) Maximum-likelihood phylogenetic tree built from the alignments of concatenated SctN and SctC amino acid sequences with a bootstrap test of phylogeny (1000 replications) with a Jones-Taylor-Thornton (JTT) correction model and visualized by iTOL. The presence of hemolysis and subgroups are shown as colored strips. T3SS2α and T3SS2β are shown in red and blue branches, respectively. (C) Same tree as B but with branch length information.

Fig. 3. T3SS2 gene clusters share a high degree of synteny. Schematic depiction of a comparison of the T3SS2 gene clusters in *V. parahaemolyticus* RIMD2210633 in comparison to the gene clusters of strains of *Providencia* (A), *Photobacterium* (B) and *Aeromonas,* and *Yersinia enterocolitica* strains (C). BLASTn alignments were performed and visualized using Easyfig. Genes encoding T3SS-related genes are highlighted in blue.

Fig. 4. Distinctive genomic locations of T3SS2 gene clusters. (A) Comparison of the T3SS2 gene cluster in *V. parahaemolyticus* RIMD2210633 with the T3SS2 gene cluster encoded in *V. mediterranei* 116-T6 plasmid and the T3SS2 gene cluster of *Grimontia hollisae* NCTC11640. (B) Schematic depiction of a comparison of the T3SS1 and T3SS2 gene clusters inserted in the vicinity of the serine tRNA in *Vibrio campbellii* strain UMTGB204. BLASTn alignments were performed and visualized using Easyfig. Genes encoding T3SS-related genes are highlighted in blue.

Fig. 5. Hierarchical clustering analysis of T3SS2-related genes identifies 6 distinct subgroups. Clustering analysis was performed using Versatile matrix visualization and analysis software MORPHEUS (one minus Pearson correlation, average linkage method). Boxes representing genes not detected, detected, or detected but divergent (less than 70% identity and 80% sequence coverage) are colored in grey, blue or red, respectively. An asterisk highlights the group of *V. anguillarum* genomes that only harbored a limited number of T3SS structural components.

Fig. 6. Differential distribution of T3SS2 effector proteins. (A) Frequency of detection of each T3SS2-related gene in each T3SS2 phylotype and Subgroup. Frequencies are color-coded and expressed as percentages. (B) Taxonomic distribution of the T3SS2 Subgroups (I-VI) in comparison to the T3SS2 phylotypes.

Fig. 7. T3SS2 Subgroup VI encodes 10 novel effector candidates. (A) Schematic representation of novel candidate highlighting the presence of predicted functional domains in different colors. (B) Taxonomic distribution of each predicted effector protein identified in bacterial genomes. Red presence, white absence. (C) Frequency of the presence of each predicted novel effector protein in the different T3SS2 subgroups (I-VI).

Frequencies are color-coded and expressed as percentages.

Table S1. Table with information regarding the presence/absence and hierarchical clustering analysis of T3SS2 components in the bacterial genome sequences (including accession numbers).

Table S2. Prediction of T3SS effector candidates within the T3SS2 subgroup VI gene clusters. Representative ORFs for each prediction are shown with the results of different prediction software, the identification of putative functional domains (InterproScan), and the amino acid sequence identity to known T3SS effector proteins from other organisms.

Fig. S1. Distribution of *Vibrio parahaemolyticus* Sequence Types (STs) in terms of the presence of T3SS2α or T3SS2β gene clusters.

Fig. S2. Gene synteny between the T3SS2 gene clusters of *V. parahaemolyticus* RIMD2210633 and the identified T3SS2 gene clusters of *Photorhabdus* species. BLASTn alignments were performed and visualized using Easyfig. Genes encoding T3SS-related genes are highlighted in blue.

Fig. S3. Only VgpA is widely conserved among bacterial species harboring T3SS2 gene clusters. The distribution matrix shows the detection of each effector protein in at least one bacterial strain in red. The total number of bacterial species in which each effector was detected is shown at the bottom of the matrix.

Fig. S4. Multiple sequence alignment of the amino acid sequence of the predicted effector BCT80_RS20435 and the secreted lipase VPA0226 and the *Salmonella* T3SS effector

protein SseJ. The alignment was performed using T-Coffee Expresso and visualized by ESPript 3.0. Amino acids within a red background correspond to positions with 100% identity; amino acids with a yellow background correspond to positions with >70% identity.

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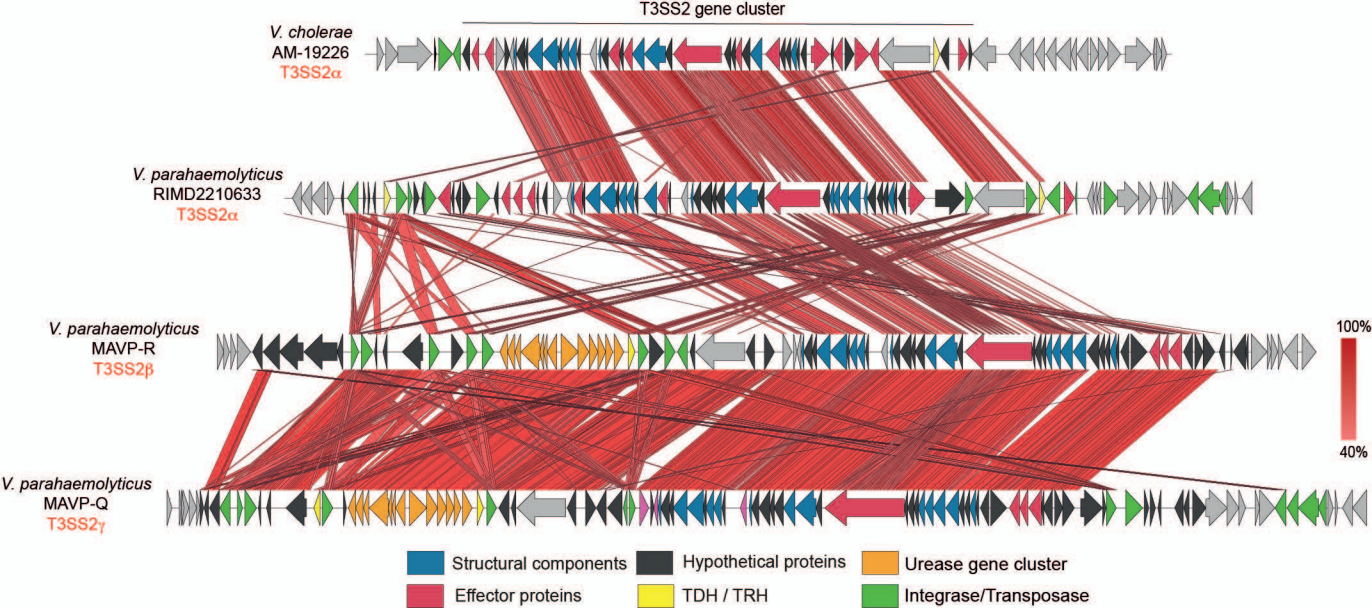
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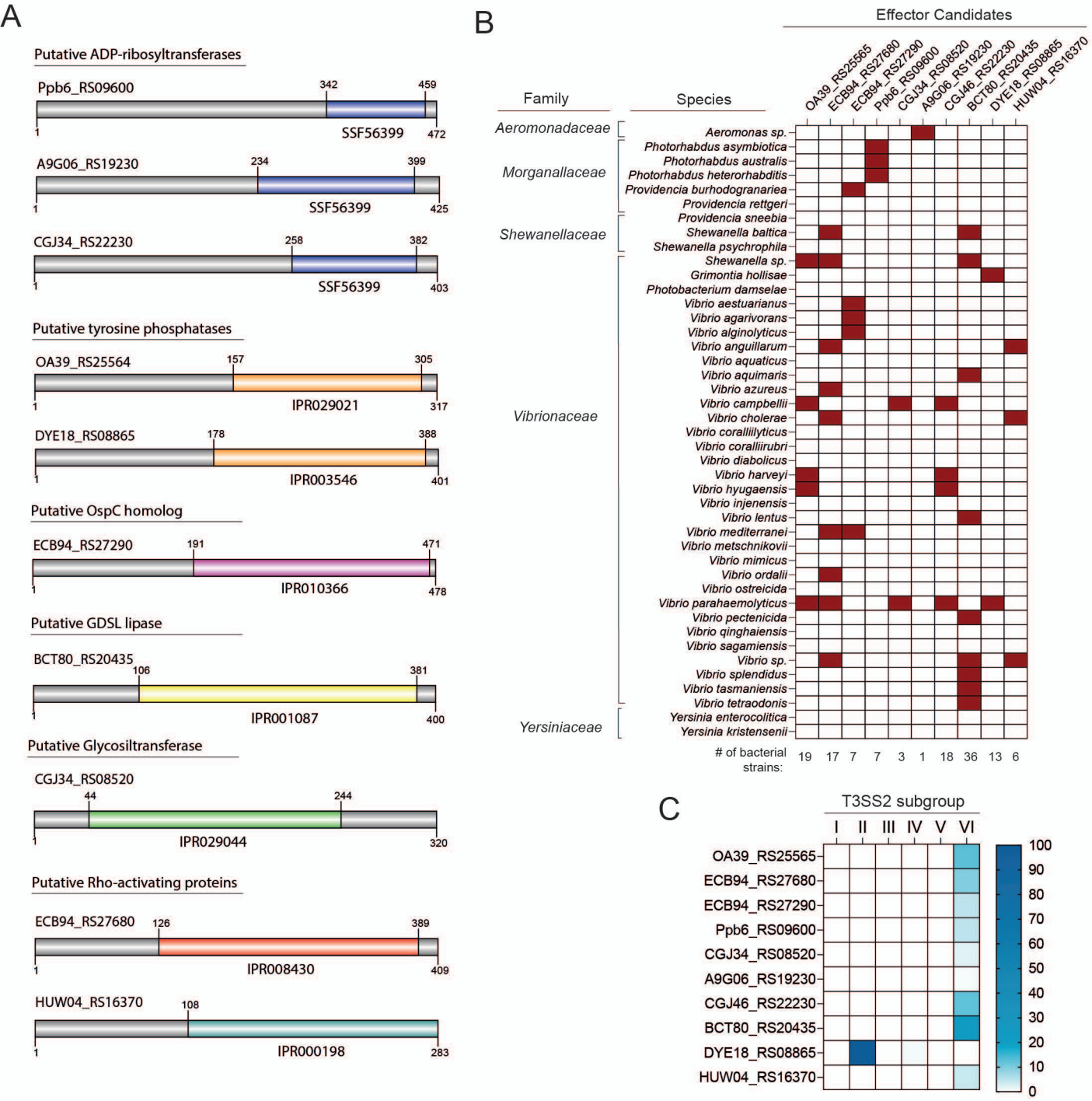
T3SS2α
V. parahaemolyticus
RIMD2210633
T3SS2α
V. parahaemolyticus
MAVP-R
T3SS2β
V. parahaemolyticus
MAVP-Q
T3SS2γ
100%
40%
Structural components
Hypothetical proteins
Urease gene cluster
Effector proteins
TDH / TRH
Integrase/Transposase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1
SSF56399
472
A9G06_RS19230
234
399
1
SSF56399
425
CGJ34_RS22230
258
382
1
SSF56399
403
Putative tyrosine phosphatases
OA39_RS25564
157
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IPR029021
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DYE18_RS08865
178
388
1
IPR003546
401
Putative OspC homolog
ECB94_RS27290
191
471
1
IPR010366
478
Putative GDSL lipase
BCT80_RS20435
106
381
1
IPR001087
400
Putative Glycosiltransferase
CGJ34_RS08520
44
244
1
IPR029044
320
Putative Rho-activating proteins
ECB94_RS27680
126
389
1
IPR008430
409
HUW04_RS16370
108
1
IPR000198
283
B
Effector Candidates
OA39_RS25565
ECB94_RS27680
ECB94_RS27290
Ppb6_RS09600
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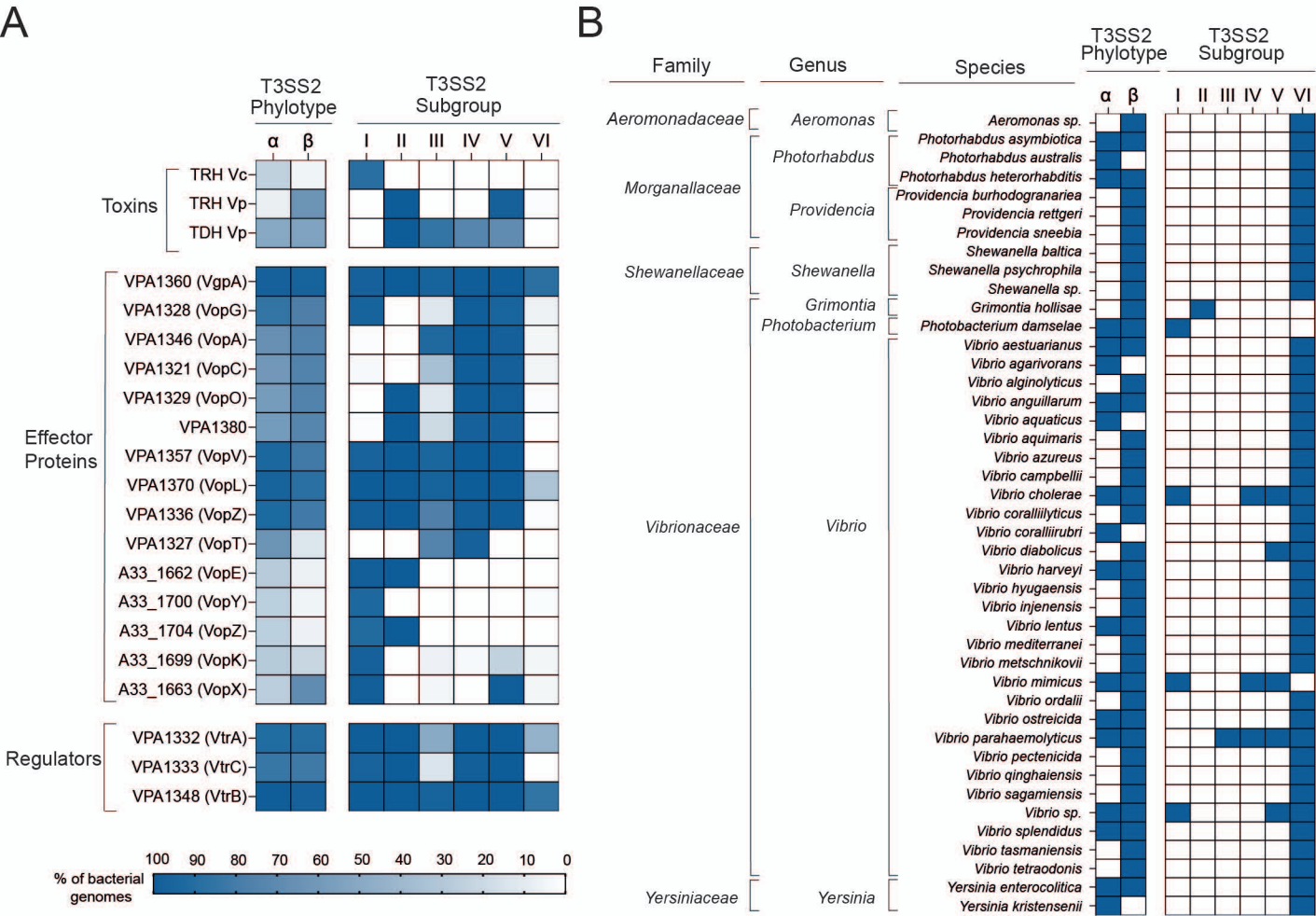
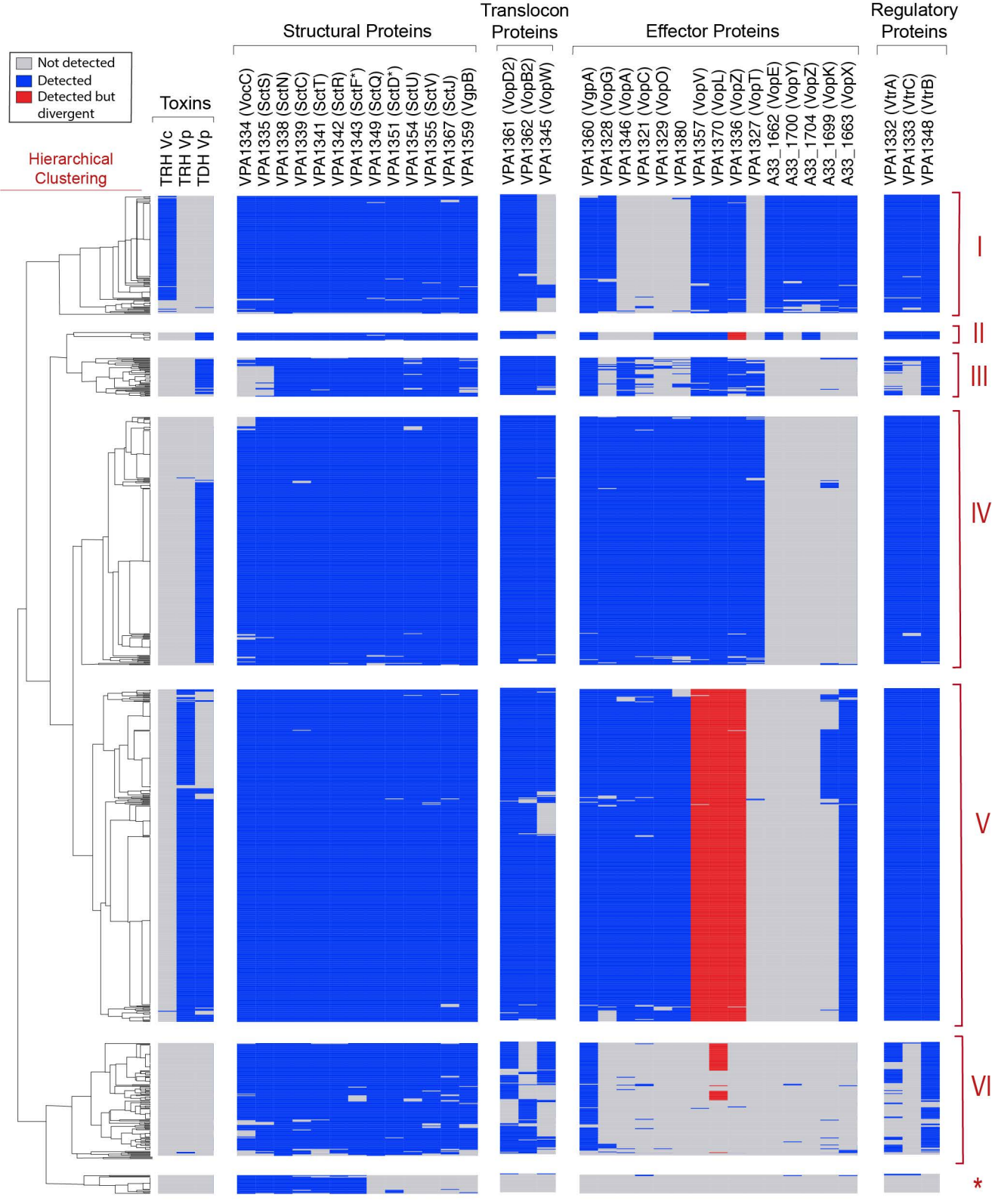
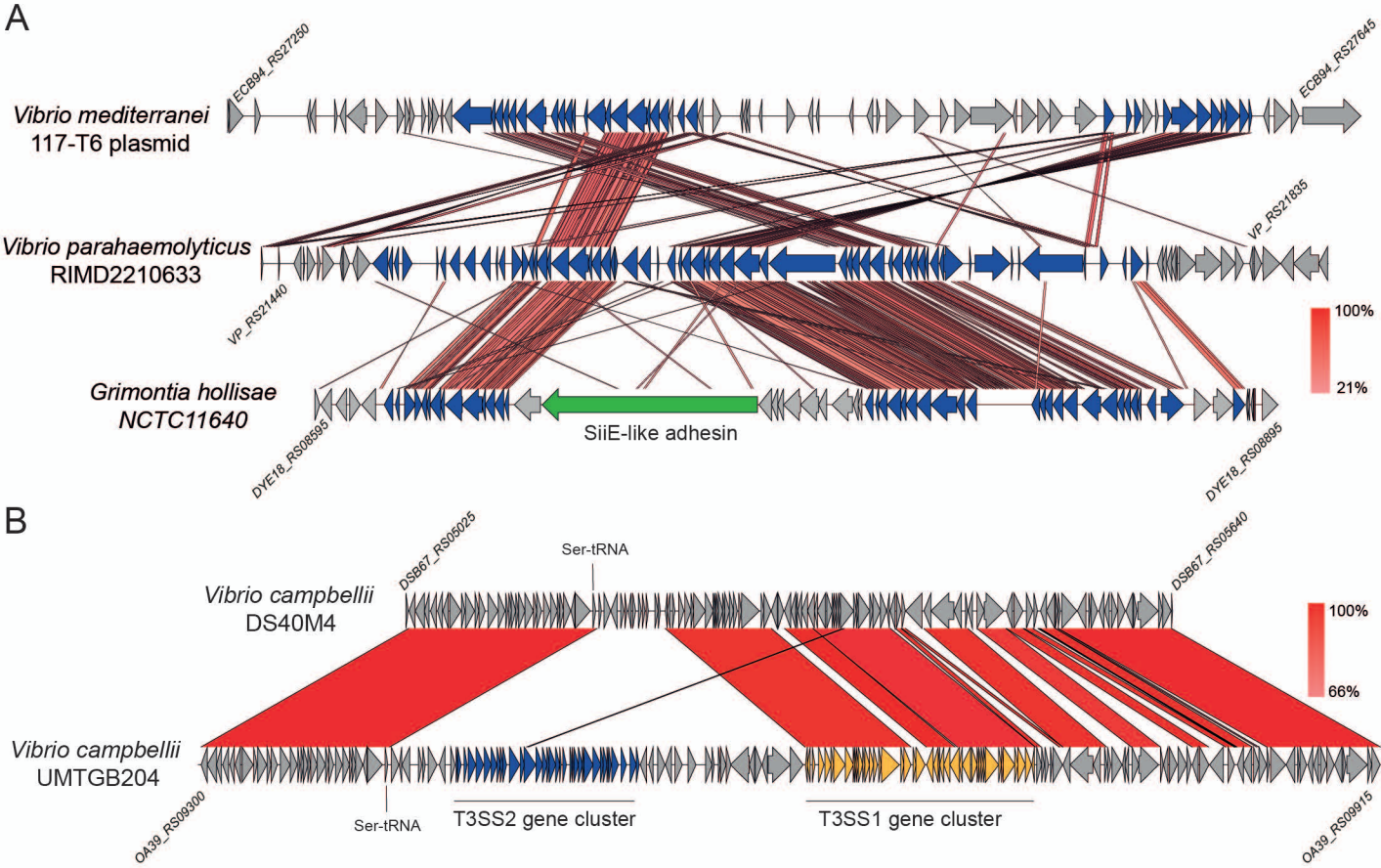
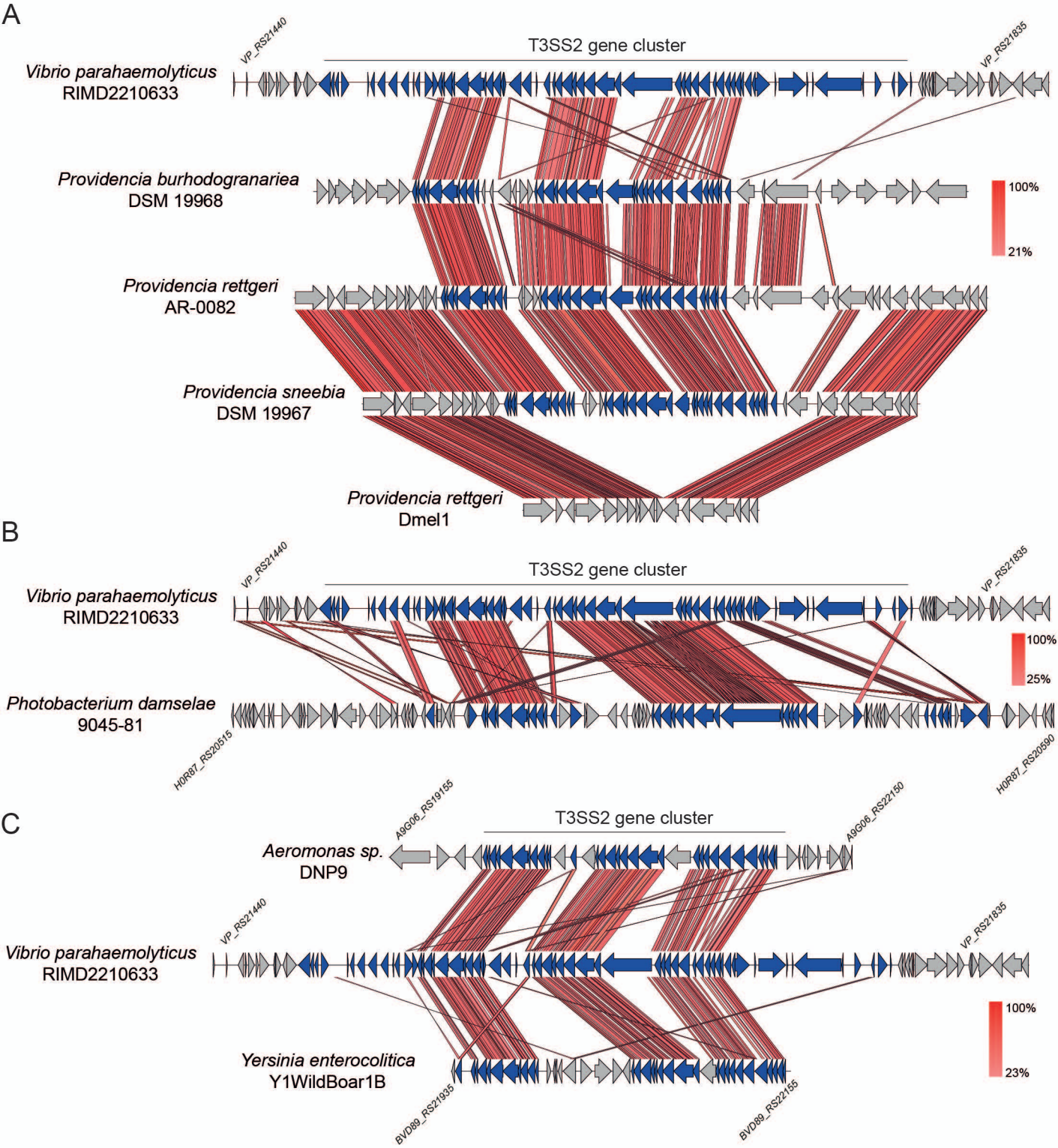
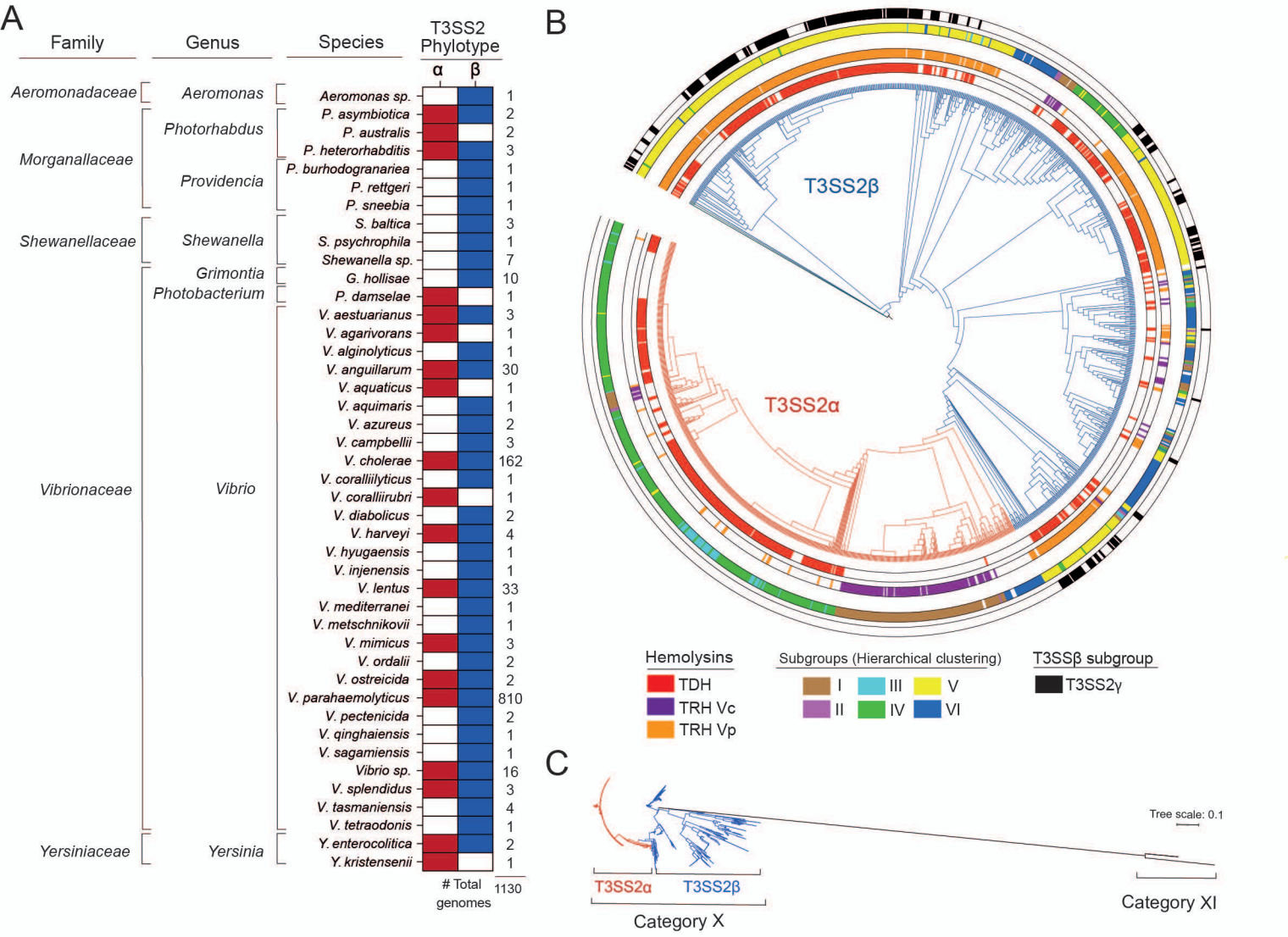
CGJ46_RS22230
BCT80_RS20435
DYE18_RS08865
HUW04_RS16370
Family
Species
Aeromonadaceae
Morganallaceae
Shewanellaceae
Vibrionaceae
Yersiniaceae
Aeromonas sp.
Photorhabdus asymbiotica
Photorhabdus australis
Photorhabdus heterorhabditis
Providencia burhodogranariea
Providencia rettgeri
Providencia sneebia
Shewanella baltica
Shewanella psychrophila
Shewanella sp.
Grimontia hollisae
Photobacterium damselae
Vibrio aestuarianus
Vibrio agarivorans
Vibrio alginolyticus
Vibrio anguillarum
Vibrio aquaticus
Vibrio aquimaris
Vibrio azureus
Vibrio campbellii
Vibrio cholerae
Vibrio coralliilyticus
Vibrio coralliirubri
Vibrio diabolicus
Vibrio harveyi
Vibrio hyugaensis
Vibrio injenensis
Vibrio lentus
Vibrio mediterranei
Vibrio metschnikovii
Vibrio mimicus
Vibrio ordalii
Vibrio ostreicida
Vibrio parahaemolyticus
Vibrio pectenicida
Vibrio qinghaiensis
Vibrio sagamiensis
Vibrio sp.
Vibrio splendidus
Vibrio tasmaniensis
Vibrio tetraodonis
Yersinia enterocolitica
Yersinia kristensenii
# of bacterial strains:
19 17 7 7 3 1 18 36 13 6
C
T3SS2 subgroup
I II III IV V VI
OA39_RS25565
ECB94_RS27680
ECB94_RS27290
Ppb6_RS09600
CGJ34_RS08520
A9G06_RS19230
CGJ46_RS22230
BCT80_RS20435
DYE18_RS08865
HUW04_RS16370
100
90
80
70
60
50
40
30
20
10
0


A
T3SS2 Phylotype
α
β
T3SS2 Subgroup
I
II
III
IV
V
VI
Toxins
TRH Vc
TRH Vp
TDH Vp
Effector Proteins
VPA1360 (VgpA)
VPA1328 (VopG)
VPA1346 (VopA)
VPA1321 (VopC)
VPA1329 (VopO)
VPA1380
VPA1357 (VopV)
VPA1370 (VopL)
VPA1336 (VopZ)
VPA1327 (VopT)
A33_1662 (VopE)
A33_1700 (VopY)
A33_1704 (VopZ)
A33_1699 (VopK)
A33_1663 (VopX)
Regulators
VPA1332 (VtrA)
VPA1333 (VtrC)
VPA1348 (VtrB)
100 90 80 70 60 50 40 30 20 10 0
% of bacterial genomes
B
Family
Genus
Species
T3SS2 Phylotype
T3SS2 Subgroup
Aeromonadaceae
Aeromonas
Aeromonas sp.
Morganallaceae
Photorhabdus
Photorhabdus asymbiotica
Photorhabdus australis
Photorhabdus heterorhabditis
Providencia
Providencia burhodogranariea
Providencia rettgeri
Providencia sneebia
Shewanellaceae
Shewanella
Shewanella baltica
Shewanella psychrophila
Shewanella sp.
Vibrionaceae
Grimontia
Grimontia hollisae
Photobacterium
Photobacterium damselae
Vibrio
Vibrio aestuarianus
Vibrio agarivorans
Vibrio alginolyticus
Vibrio anguillarum
Vibrio aquaticus
Vibrio aquimaris
Vibrio azureus
Vibrio campbellii
Vibrio cholerae
Vibrio coralliilyticus
Vibrio coralliirubri
Vibrio diabolicus
Vibrio harveyi
Vibrio hyugaensis
Vibrio injenensis
Vibrio lentus
Vibrio mediterranei
Vibrio metschnikovii
Vibrio mimicus
Vibrio ordalii
Vibrio ostreicida
Vibrio parahaemolyticus
Vibrio pectenicida
Vibrio qinghaiensis
Vibrio sagamiensis
Vibrio sp.
Vibrio splendidus
Vibrio tasmaniensis
Vibrio tetraodonis
Yersiniaceae
Yersinia
Yersinia enterocolitica
Yersinia kristensenii


Not detected
Detected
Detected but divergent
Hierarchical Clustering
Toxins
TRH Vc
TRH Vp
TDH Vp
Structural Proteins
VPA1334 (VocC)
VPA1335 (SctS)
VPA1338 (SctN)
VPA1339 (SctC)
VPA1341 (SctT)
VPA1342 (SctR)
VPA1343 (SctF*)
VPA1349 (SctQ)
VPA1351 (SctD*)
VPA1354 (SctU)
VPA1355 (SctV)
VPA1367 (SctJ)
VPA1359 (VgpB)
Translocon Proteins
VPA1361 (VopD2)
VPA1362 (VopB2)
VPA1345 (VopW)
Effector Proteins
VPA1360 (VgpA)
VPA1328 (VopG)
VPA1346 (VopA)
VPA1321 (VopC)
VPA1329 (VopO)
VPA1380
VPA1357 (VopV)
VPA1370 (VopL)
VPA1336 (VopZ)
VPA1327 (VopT)
A33_1662 (VopE)
A33_1700 (VopY)
A33_1704 (VopZ)
A33_1699 (VopK)
A33_1663 (VopX)
Regulatory Proteins
VPA1332 (VtrA)
VPA1333 (VtrC)
VPA1348 (VtrB)
I
II
III
IV
V
VI
*


A
Vibrio mediterranei
117-T6 plasmid
ECB94_RS27250
ECB94_RS27645
Vibrio parahaemolyticus
RIMD2210633
VP_RS21440
VP_RS21835
Grimontia hollisae
NCTC11640
DYE18_RS08595
DYE18_RS08805
SiiE-like adhesin
100%
21%
B
Vibrio campbellii
DS40M4
DSB67_RS05025
DSB67_RS05640
Ser-tRNA
100%
66%
Vibrio campbellii
UMTGB204
OA39_RS09300
OA39_RS09915
Ser-tRNA
T3SS2 gene cluster
T3SS1 gene cluster


A
T3SS2 gene cluster
VP_RS21440
VP_RS21835
Vibrio parahaemolyticus
RIMD2210633
Providencia burhodogranariea
DSM 19968
Providencia rettgeri
AR-0082
Providencia sneebia
DSM 19967
Providencia rettgeri
Dmel1
100%
21%
B
T3SS2 gene cluster
VP_RS21440
VP_RS21835
Vibrio parahaemolyticus
RIMD2210633
Photobacterium damselae
9045-81
H0R87_RS20515
H0R87_RS20590
100%
25%
C
T3SS2 gene cluster
A9G06_RS19155
A9G06_RS22150
Aeromonas sp.
DNP9
VP_RS21440
VP_RS21835
Vibrio parahaemolyticus
RIMD2210633
Yersinia enterocolitica
Y1WildBoar1B
BVD89_RS21935
BVD89_RS22155
100%
23%


A
Family
Genus
Species
T3SS2 Phylotype
α
β
Aeromonadaceae
Aeromonas
Aeromonas sp. 1
Morganallaceae
Photorhabdus
P. asymbiotica 2
P. australis 2
P. heterorhabditis 3
Providencia
P. burhodogranariea 1
P. rettgeri 1
P. sneebia 1
Shewanellaceae
Shewanella
S. baltica 3
S. psychrophila 1
Shewanella sp. 7
Grimontia
G. hollisae 10
Photobacterium
P. damselae 1
Vibrionaceae
Vibrio
V. aestuarianus 3
V. agarivorans 1
V. alginolyticus 1
V. anguillarum 30
V. aquaticus 1
V. aquimaris 1
V. azureus 2
V. campbellii 3
V. cholerae 162
V. coralliilyticus 1
V. coralliirubri 1
V. diabolicus 2
V. harveyi 4
V. hyugaensis 1
V. injenensis 1
V. lentus 33
V. mediterranei 1
V. metschnikovii 1
V. mimicus 3
V. ordalii 2
V. ostreicida 2
V. parahaemolyticus 810
V. pectenicida 2
V. qinghaiensis 1
V. sagamiensis 1
Vibrio sp. 16
V. splendidus 3
V. tasmaniensis 4
V. tetraodonis 1
Yersiniaceae
Yersinia
Y. enterocolitica 2
Y. kristensenii 1
Total genomes 1130
B
T3SS2β
T3SS2α
Hemolysins
TDH
TRH Vc
TRH Vp
Subgroups (Hierarchical clustering)
I
II
III
IV
V
VI
T3SSβ subgroup
T3SS2γ
C
Tree scale: 0.1
T3SS2α
T3SS2β
Category X
Category XI