



An onboard checking mechanism ensures effector delivery of the type VI secretion system in *Vibrio cholerae*

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The type VI secretion system (T6SS) is a lethal yet energetically costly weapon in gram-negative bacteria. Through contraction of a long sheath, the T6SS ejects a few copies of effectors accompanied by hundreds of structural carrier proteins per delivery. The few ejected effectors, however, dictate T6SS functions. It remains elusive how the T6SS ensures effector loading and avoids futile ejection. Here, by systematically mutating the active sites of 3 *Vibrio cholerae* effectors, TseL, VasX, and VgrG3, we show that the physical presence but not their activities is crucial for T6SS assembly. We constructed catalytic mutants of TseL and VgrG3 and truncated VasX mutants. These mutations abolished the killing of the effector-cognate immunity mutants. We determined that the VasX-mediated antimicrobial activity is solely dependent on the C-terminal colicin domain. Removal of the colicin domain abolished VasX secretion and reduced T6SS assembly, while deletion of the colicin internal loop abolished its toxicity but had little effect on secretion and assembly. The triple effector-inactive mutant maintains an active T6SS that is capable of delivering chimeric VgrG, PAAR, and TseL proteins fused with a cargo nuclease, indicating effector activities are not required for T6SS assembly or penetration into the cytosol of recipient cells. Therefore, by recruiting effectors as critical components for T6SS assembly, it represents an effective onboard checking mechanism that ensures effectors are loaded in place to prevent futile secretion. Our study also demonstrates a detoxified secretion platform by inactivating native effector activities that could translocate engineered cargo proteins via multiple routes.

protein secretion | interspecies interaction | effector | synthetic biology

Recent progress in microbiome studies has greatly expanded our traditional view of microbes in human health from infectious disease-focused to almost every aspect ranging from mental health to autoimmune diseases (1, 2). It is important not only to identify what microbes are present but also to understand how they exist and interact in complex communities. Of the diverse tools microbes have evolved for interaction, the type VI protein secretion system (T6SS) is one with wide distribution and diverse functions in microbial competition and host-microbe interactions in gram-negative commensals as well as pathogens (3–6).

Vibrio cholerae is a waterborne pathogen that causes cholera, an acute infectious disease from which patients develop severe diarrhea and dehydration (7). Cholera is a global public health threat with an estimated ~3 million annual cases and ~1.3 billion people at risk in 69 cholera-endemic countries (8). In addition to the key virulence factors cholera toxin and toxin-coregulated pili, *V. cholerae* has also acquired the T6SS that functions as a molecular weapon to inject antibacterial and antieukaryotic effectors into target cells through direct contact (4, 5, 9, 10). The T6SS is activated during infection in the host and facilitates *V. cholerae* colonization by outcompeting host commensal microbiota (11–13).

Belonging to the contractile injection systems (14), the T6SS consists of an intracellular double-tubular structure that ejects

the inner needle outward through contraction of the outer sheath (15–17). The needle and the sheath are made of hundreds of stacks of Hcp and VipA/VipB (TssB/TssC) proteins, respectively (15–17). The tip of the needle carries a sharpening trimeric VgrG spike and a cone-shaped PAAR proposed for piercing through target cells (18, 19). *V. cholerae* encodes 3 VgrG proteins, of which VgrG1 and VgrG3 carry extended functional domains and display actin cross-linking and cell wall-degrading activities, respectively (9, 18, 20). VgrG1 and VgrG2 also serve as carrier proteins for their respective antibacterial effectors, TseL and VasX, both targeting the membrane (21, 22). TseL carries a lipase domain, and VasX carries an N-terminal predicted Pleckstrin-homology (PH) domain for interacting with phosphorylated membrane lipids and a C-terminal predicted colicin domain (9, 23–25). Whether the C-terminal colicin domain is solely responsible for the antibacterial activity is not known. Each antibacterial effector has a cognate immunity protein to confer self-protection. In addition, chaperone proteins are needed for VgrG-mediated delivery of TseL and VasX (21, 22). *V. cholerae* possesses another cell wall-targeting effector TseH (26). However, its role as a secreted effector is unclear since TseH has no effect in killing *Escherichia coli* or *V. cholerae* mutant lacking its cognate immunity gene *tsiH* (26).

As a protein delivery system, the diverse physiological functions of T6SS largely depend on its secreted effector proteins in different organisms (10, 18, 27–30). In addition, effectors also contribute to T6SS assembly because deletion of effector genes $\Delta vgrG3/\Delta vasX$ and $\Delta tseL/\Delta vgrG3/\Delta vasX$ abolished Hcp secretion and sheath assembly in *V. cholerae* (9, 23, 31). In contrast to

Significance

How to ensure effectors are loaded efficiently is a key question to understanding protein secretion systems. Our study demonstrates that the type VI secretion system (T6SS) of *Vibrio cholerae* requires the physical presence of effectors as a prerequisite for assembling a functional secretion apparatus, representing a distinct posttranslational energy-saving strategy that may be conserved among the diverse T6SS systems in gram-negative bacteria.

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the evolved VgrG, Hcp, and PAAR effectors (18, 19, 32), it is less intuitive to envision how dedicated effectors without conserved T6SS structural domains, such as TseL and VasX, could affect the T6SS assembly. Indeed, deletion of all known effectors in *Agrobacterium tumefaciens* and *Acinetobacter baylyi* ADP1 has little effect on T6SS secretion (33, 34). It is not known whether the T6SS requirement for effectors is dependent on their activities or physical structures. For example, the T6SS of *Acinetobacter* requires a membrane-associated peptidoglycan hydrolase TagX to facilitate formation of the transenvelope TssJLM complex of the T6SS (35). Since *V. cholerae* lacks a TagX homolog, how the T6SS of *V. cholerae* assembles its transenvelope complex across the cell wall remains elusive. Considering that both TseL and VgrG3 are capable of reaching the periplasm when expressed in the cytosol (36), could *V. cholerae* effectors fulfill such TagX-like function through their membrane/cell wall-targeting activities?

To address this question, here we constructed catalytically inactive effector mutants to abolish their functions while minimizing perturbation to their structures in *V. cholerae*. We report that T6SS does not require effector activities for T6SS assembly or cytosol-to-cytosol penetration. In addition, the effector-inactive T6SS could deliver engineered VgrG, PAAR, and effector carrying a C-terminal cargo nuclease, demonstrating that PAAR and effectors are also delivered into the cytosol by the T6SS and can serve as carriers. Therefore, we conclude that the T6SS of *V. cholerae* requires the physical presence but not the antimicrobial activities of effectors. This represents an effective energy-conserving strategy to prevent futile secretion of structural proteins.

Results

Construction of Chromosomal Effector-Inactive Mutants. To determine how effectors contribute to T6SS assembly in *V. cholerae*, we decided to construct combinatorial deletion mutants and functionally inactive mutants of 3 known antibacterial effectors, VgrG3, TseL, and VasX (9) (Fig. 1A). To monitor T6SS assembly, we constructed all mutants in a fluorescence-labeled sheath VipAmCherry2 V52 strain that maintains wild-type level T6SS activities (17, 37). Since we have previously identified active sites of TseL and VgrG3 (9), we constructed chromosomally encoded inactive mutants, TseL^{D425A} and VgrG3^{D842A}, accordingly. Unlike TseL and VgrG3 possessing enzymatic activities, VasX carries a C-terminal colicin domain without a catalytic pocket and it is unknown whether the observed antibacterial activity of VasX is solely dependent on this colicin domain (Fig. 1A). To inactivate VasX, we constructed a mutant VasX^{ΔCd} by deleting the colicin domain.

We then tested catalytic mutants competing with their respective immunity gene mutants and *E. coli* to compare their

antibacterial activities (Fig. 1B). Competition assays show that tseL^{D425A}, vgrG^{D842A}, and vasX^{ΔCd} individual mutants failed to outcompete their specific immunity mutants but effectively killed *E. coli* and *V. cholerae* mutants lacking other immunity protection. Interestingly, *E. coli* could be killed by double-effector mutants of tseL^{D425A}/vasX^{ΔCd} or tseL^{D425A}/vgrG3^{D842A} but not by vgrG3^{D842A}/vasX^{ΔCd}. The vgrG3^{D842A}/vasX^{ΔCd} double mutant could kill the TseL-specific ΔtsiV1 mutant, indicative of an active T6SS capable of delivering TseL. As expected, the triple mutant exhibited no difference from the ΔvasK mutant in all competition assays, indicating the loss of T6SS-mediated toxicity.

VgrG3 and TseL Enzymatic Activities Are Not Required for T6SS Assembly.

As VgrG3 is a T6SS structural protein, we then investigated whether the loss of VgrG3 function is responsible for abolishing T6SS activities in the triple effector deletion ΔtseL/ΔvgrG3/ΔvasX mutant (9). As expected, fluorescence microscopy imaging of T6SS sheath formation reveals that the T6SS was actively assembled in wild-type cells but not in the triple effector deletion mutant ΔtseL/ΔvasX/ΔvgrG3 (Fig. 2A and B). Deletion of ΔtseL/ΔvasX significantly reduced T6SS assembly activities to about 25% of wild-type level (Fig. 2C and SI Appendix, Fig. S1). Catalytic inactivation of VgrG3 (VgrG3^{D842A}) slightly reduced T6SS assembly in the ΔtseL/ΔvasX mutant and had little effect in wild type (Fig. 2D and E and SI Appendix, Fig. S1). We then tested the chromosomally inactivated tseL^{D425A}/vgrG3^{D842A} mutant (Fig. 2F). This mutant exhibited wild-type-level T6SS activities as the vgrG3^{D842A} mutant, indicating the catalytic activities of TseL and VgrG3 are not required for T6SS transenvelope complex assembly despite their capability of periplasmic translocation (36).

Deletion of the Colicin Domain of VasX Reduces T6SS Assembly.

VasX deletion has been shown to reduce T6SS assembly but the mechanism is elusive (31, 38). We then compared T6SS assembly in mutants lacking the colicin domain. The vasX^{ΔCd} mutant and the triple tseL^{D425A}/vgrG3^{D842A}/vasX^{ΔCd} mutant both show about 50% reduced T6SS assemblies relative to the wild type and the double tseL^{D425A}/vgrG3^{D842A} mutant, respectively (Fig. 2G and H). These data suggest that the colicin domain-mediated toxicity is critical for T6SS assembly or, alternatively, the deletion of colicin domain causes structural deformation of VasX affecting T6SS assembly. Nonetheless, the active T6SS in those mutants suggests effector activities are not required for T6SS assembly.

Construction of a Colicin-Inactive and Secreted VasX Mutant.

Protein sequence analysis using the HMMER program predicted 3 transmembrane regions within the colicin domain (Fig. 3A). To further test whether the colicin-mediated activity is critical

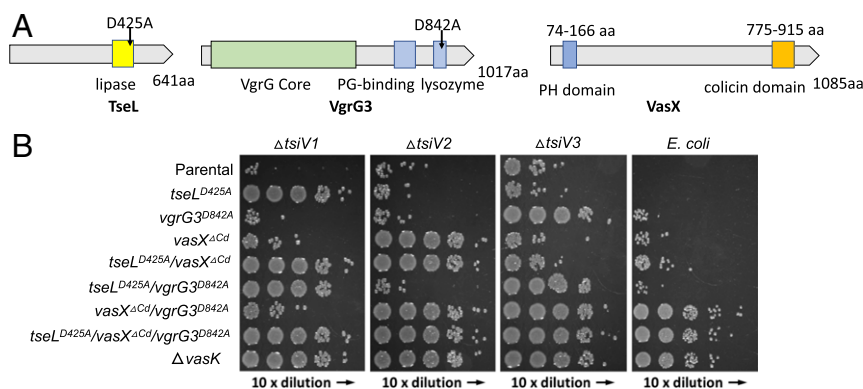


Fig. 1. Chromosomal inactivation of effectors. (A) Predicted domains and mutation sites in *V. cholerae* effectors. (B) Bacterial competition assays. Killer strains are indicated on the left and prey strains on the top. A tetracycline-resistant strain CC114 is used as *E. coli* prey. Representative data from 3 independent assays are shown.

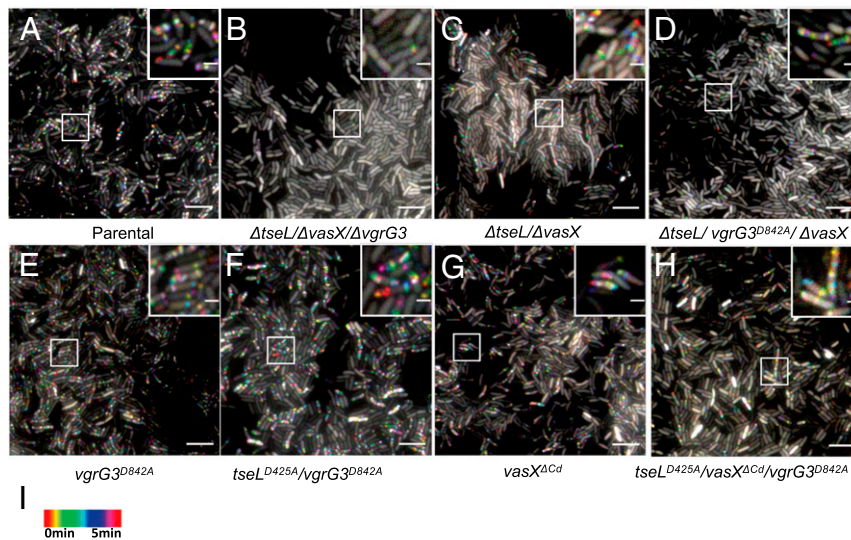


Fig. 2. Fluorescence microscopy of T6SS assembly in effector mutants. (A–H) Time-lapse imaging of VipA-mCherry2 signal was captured every 10 s for 5 min and temporally color-coded. Representative images are shown with a close-up of a selected region as an inset. A 40- × 40-μm representative field of cells with a 3× magnified 5- × 5-μm inset (marked by box) is shown. Genotypes are indicated at the bottom. (Scale bars: large field of view, 5 μm and insets, 1 μm.) (I) Color scale used to temporally color-code the VipA-mCherry2 signal.

for T6SS assembly, we deleted the second transmembrane loop within the colicin domain to inactivate VasX toxicity while minimizing structural changes. The resultant VasX^{ΔC16} mutant

(lacking A852 to F867) showed no toxicity against the VasX immunity mutant $\Delta tsiV2$ similar to the $\Delta vasK$ mutant, suggesting VasX^{ΔC16} is inactivated (Fig. 3B).

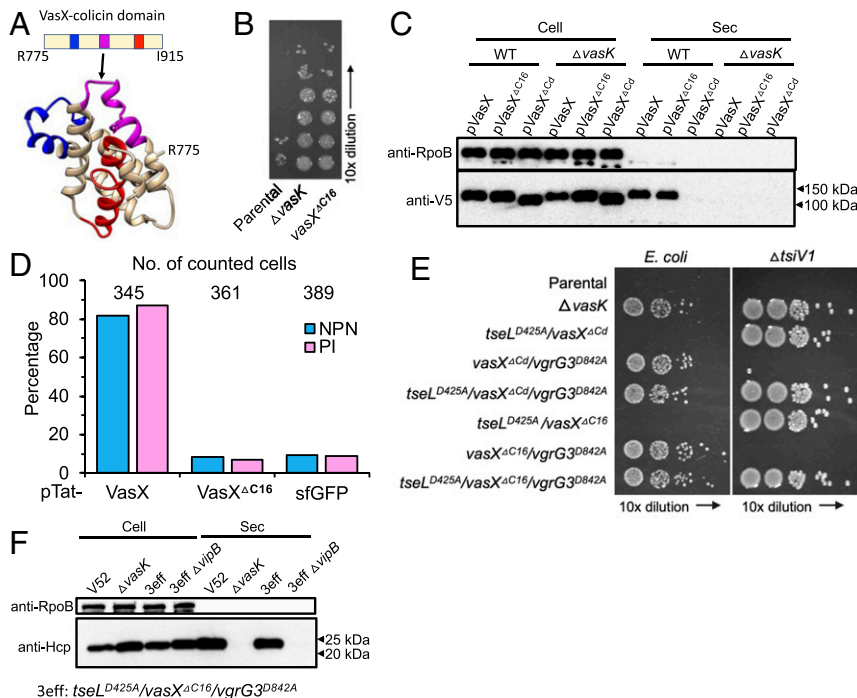


Fig. 3. Deletion of a colicin loop abolishes VasX toxicity but maintains secretion. (A) Predicted structural model of the colicin domain based on template 3FEW, with 13% identity and 84.3% confidence. Three predicted transmembrane regions within the colicin domain are highlighted in different colors. The loop within the second transmembrane region (pink) between A852 and F867 is deleted to result in VasX^{ΔC16}. (B) Competition assay using the $\Delta tsiV2$ mutant as prey. (C) Secretion of VasX and its derivative mutants. Secretion of 3xV5 epitope-tagged VasX and its derivative mutants was detected using anti-V5 antiserum. (D) Membrane permeability and live–dead staining using NPN and PI fluorescence dyes. VasX and VasX^{ΔC16} were induced in the periplasm using a Tat signal. Cells exhibiting NPN-positive and PI-positive are likely dead cells or cells with damaged membranes. Induction of sfGFP serves as a background control. See also *SI Appendix, Fig. S2* for images. (E) Bacterial competition assay. Killers are indicated on the left and prey on the top. *E. coli* CC114 was used to test the overall antibacterial toxicity and $\Delta tsiV1$ was used to specifically test TseL secretion in the activity mutant defective in both VasX and VgrG3. (F) Western blot analysis of Hcp secretion as an indicator of T6SS activity. Comparable secretion was found between wild-type V52 and the $tseL^{D425A}/vasX^{\Delta C16}/vgrG3^{D842A}$ mutant. In C and F, the RNA polymerase beta-subunit RpoB serves as a loading and cell lysis control.

To confirm that the loss of killing of the *vasX*^{ΔC16} mutant is due to VasX inactivation but not failed delivery, we performed secretion assays expressing plasmid-borne VasX and its inactive mutants in wild type and the *ΔvasK* mutant. While no secretion of VasX^{ΔCd} was detected, wild-type VasX and VasX^{ΔC16} were secreted at comparable levels (Fig. 3C), indicating VasX^{ΔC16} but not VasX^{ΔCd} can be recognized as a T6SS substrate similar to wild-type VasX.

Although VasX has been shown to cause increased membrane permeability (23), whether this depends solely on its C-terminal activity or also requires its N-terminal lipid-binding PH domain has not been determined. We then compared the membrane permeability and cell death using the fluorescent probes 1-N-phenylanthranilic acid (NPN) and propidium iodide (PI) in cells periplasmically expressing VasX and its VasX^{ΔC16} using the twin-arginine translocation signal sequence (Tat) (26, 39). NPN interacts with membrane phospholipids and PI intercalates DNA, respectively, but both are not membrane-permeable except in damaged cells. Indeed, expression of VasX showed considerable cell damage, while the *vasX*^{ΔC16} mutation reduced both NPN- and PI-positive cells to background levels, supporting that the C-terminal colicin domain dictates VasX toxicity (Fig. 3D and *SI Appendix*, Fig. S2).

The Triple Effector-Inactive Mutant Actively Assembles Sheath Structures. We next constructed VasX^{ΔC16} in combination with other effector mutations and tested their activities in bacterial competition assays (Fig. 3E). The triple effector mutant *tseL*^{D425A}/*vasX*^{ΔC16}/*vgrG3*^{D842A} exhibited no difference from the *ΔvasK* mutant in competition assays, indicating abolished T6SS toxicity. Notably, the *vasX*^{ΔC16}/*vgrG3*^{D842A} mutant efficiently killed the *ΔtsiVI* prey, suggesting TseL is delivered, but it failed to kill *E. coli*. Consistent results were also obtained using the *vasX*^{ΔCd}/*vgrG3*^{D842A} mutant. These data suggest that TseL might have evolved to selectively target *V. cholerae* but not *E. coli*.

To test whether the *tseL*^{D425A}/*vasX*^{ΔC16}/*vgrG3*^{D842A} is T6SS-active, we monitored sheath assembly by microscopy and Hcp secretion by Western blot analysis (Fig. 3F and *SI Appendix*, Fig. S1). Indeed, T6SS was actively assembled and secretion of Hcp was equivalent to wild-type levels in the *tseL*^{D425A}/*vasX*^{ΔC16}/*vgrG3*^{D842A} mutant.

Distribution of VasX in Gram-Negative Bacteria. Our data highlight the importance of effector presence for T6SS assembly. Since TseL is known to belong to a large lipase family (25), we next investigated the distribution of VasX using HMMER to search for homologs of VasX in the UniProtKB database (40). By visually inspecting the pairwise hit positions and setting a stringent cutoff E-value of e-51, we reduced the number of 1,026 hits to a smaller set of 198 hits. Neighbor-joining clustering of the identified homologs demonstrates that the majority of VasX homologs are found in the families of *Vibrionaceae*, *Aeromonadaceae*, and *Pseudomonadaceae* with representative species including the bioluminescent *Aliivibrio fischeri* and *Photobacterium damsela*, *Aeromonas hydrophila*, and plant pathogens *Pseudomonas syringae* and *Pseudomonas amygdali* (*SI Appendix*, Fig. S3). We constructed a maximum-likelihood phylogenetic tree of 16 representative homologs using the *E. coli* colicin S4 protein as the outgroup and compared their gene clusters (Fig. 4). Thirteen VasX homologs are encoded in clusters containing upstream genes coding for the conserved Tec chaperone (DUF4123) (21) and VgrG proteins. Two VasX homologs were found in the strain *Salinivibrio* sp. IB872, suggesting a duplication event. In *Vibrio spartinae*, a gene of unknown function is inserted between *vgrG* and its downstream chaperone gene, which is reminiscent of the chaperone-cochaperone pair upstream of an H2-T6SS-dependent effector TseT in *Pseudomonas aeruginosa* (41). Comparison of *A. hydrophila* and its closely related *Aeromonas dhakensis* reveals a PAAR gene between *hcp* and *vgrG* in

A. dhakensis. The copresence of *vgrG-tec-vasX* suggests that VasX secretion might be also tightly linked to T6SS assembly in those *Vibrio*, *Pseudomonas*, and other distantly related species.

Depth of T6SS Penetration Is Independent of Effector Activities. Finally, we asked whether effector activities are required for T6SS penetration into the cytosol of recipient cells. We employed a recently constructed chimeric VgrG3-NucSe1 nuclease in which the native lysozyme domain of VgrG3 is replaced with a nuclease domain NucSe1 (36). Bacterial competition assays show that the *tseL*^{D425A}/*vasX*^{ΔC16}/*vgrG3*^{D842A} mutant could deliver VgrG3-NucSe1 and kill prey cells except those expressing the specific immunity protein to NucSe1 (Fig. 5 and *SI Appendix*, Fig. S4). Therefore, the T6SS penetration into the cytosol is independent of effector activity but driven by the physical force of sheath contraction.

We next tested whether the NucSe1 cargo could also be delivered by the tip protein PAAR and effectors as fusion proteins. In contrast to VgrG and Hcp proteins (31), cytosol-to-cytosol delivery for the 2 PAAR proteins and membrane-targeting effectors TseL and VasX in *V. cholerae* has not been demonstrated. Using competition assays, we found that chimeric PAAR1(VCA0105), PAAR2 (VCA0284), and TseL^{D425A} could deliver NucSe1, while VasX^{ΔC16} showed a minor effect (Fig. 5 and *SI Appendix*, Fig. S4). These results not only indicate that T6SS-delivered PAAR proteins and TseL can reach the cytosol but also demonstrate the potential of using the *tseL*^{D425A}/*vasX*^{ΔC16}/*vgrG3*^{D842A} mutant as a detoxified delivery platform to translocate cargo proteins into recipient cells via multiple routes.

Discussion

T6SS assembly and secretion is energetically costly. Assembling a 1-μm-long sheath needle (average length in *V. cholerae*) requires 260 rings of hexameric VipA/B-Hcp subunits (16). Assuming sheath contraction ejects the whole Hcp needle out, each contraction will cost 1,560 molecules of Hcp to accompany the delivery of a few molecules of effectors (Fig. 6A). Therefore, how could cells prevent futile delivery of T6SS, that is, T6SS secretion without any effectors loaded? Our results demonstrate an onboard checking mechanism by which the physical presence of effectors is required for T6SS assembly in *V. cholerae*, thereby ensuring effectors are loaded for each T6SS ejection (Fig. 6B).

In addition to Hcp and effectors, each delivery also secretes a heterotrimeric VgrG spike complex (18, 42). This is evidenced by the fact that lacking VgrG1 or VgrG3 individually has little effect on T6SS functions but lacking both VgrG1 and VgrG3 severely reduces T6SS formation; by contrast, deletion of VgrG2 completely abolishes T6SS activities (18, 31, 43). Therefore, each delivery is expected to translocate at least 1 molecule of VgrG2 with 1 or 2 molecules of VgrG1/VgrG3 or both. Accordingly, considering that VasX secretion is VgrG2-dependent and TseL is VgrG1-dependent and that deletion of *vasX* and *vgrG3* severely impairs assembly (9), it is likely that each secretion event delivers 1 or 2 VgrG2-VasX with VgrG3 and/or with VgrG1-TseL (Fig. 6B). There are 2 PAAR proteins encoded by *V. cholerae*. Although PAAR proteins are critical for the T6SS in *A. baylyi*, they are dispensable in *V. cholerae* (19). The carrier for TseH secretion is currently unclear, but it is likely dependent on its upstream gene product PAAR VCA0284.

How effectors contribute to T6SS assembly remains to be elucidated. Considering the VgrG dependence of TseL and VasX (9, 23), it is plausible that the presence of effector-VgrG interaction provides a more stable VgrG-trimeric complex that is required for the sheath-needle assembly (18, 42, 44). Indeed, when we used a noncontractile sheath construct (VipA-N3) to image assembly in the triple effector deletion mutant, long sheaths were

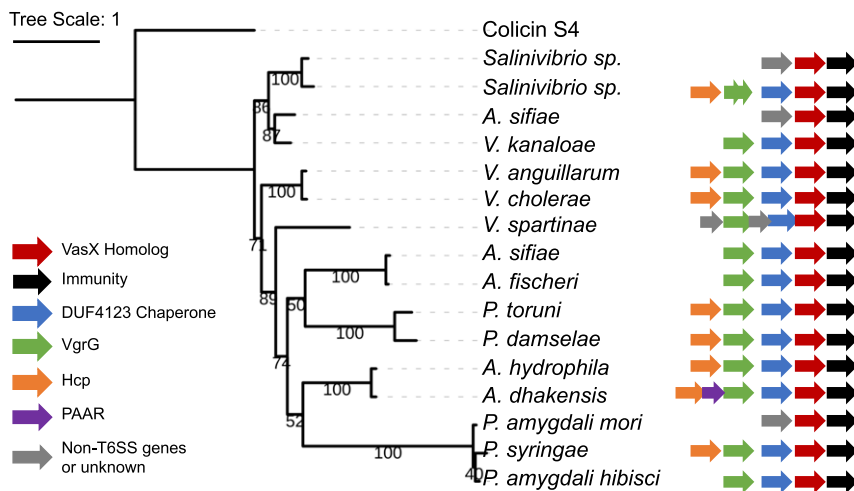


Fig. 4. Maximum likelihood phylogeny of VasX homologs and their gene clusters. Sixteen representative VasX homologs were aligned by MUSCLE and phylogeny (bootstrap = 100) was generated by PhyML using the MPI Bioinformatics Toolkit with default settings. Bootstrap value for each branch is indicated. *E. coli* colicin S4 protein, the structural template used for VasX modeling, serves as an outgroup. Gene clusters are retrieved from National Center for Biotechnology Information and curated. The size of each gene is not to scale.

observed suggesting effectors are not required for assembling the highly stable noncontractile sheath (*SI Appendix, Fig. S5*). When T6SS-delivered proteins enter the recipient cells, the interacting VgrG–effector complex should disassociate quickly to find their destined targets. Therefore, the intermolecular interaction is expected to be weak rather than strong. Stabilization of VgrG–effector complex may also require chaperone proteins carrying conserved domains (DUF4123, DUF1795, and DUF2169) that have now been identified in many T6SS species (41, 45–48). Because chaperones are not cosecreted, the delivered VgrG complex decorated with effectors might be quickly destabilized after entering recipient cells. This hypothesis can be tested by tracking the fate of delivered protein complex directly through imaging. However, this is a considerable technical challenge since it would require

superresolution live-cell microscopy with millisecond temporal resolution (49).

VasX is a transkingdom effector targeting both eukaryotic amoeba and bacterial cells (9, 23, 24). Here we show that its C-terminal colicin domain is responsible for its antibacterial activity. Interestingly, despite that the domain is only 13% of the full-length protein at the C-terminal end, deletion of this domain abolishes VasX secretion, highlighting its critical role in T6SS assembly. Notably, a recent study quantifying T6SS protein levels reports that VasX is highly unstable and prone to degradation (38). To inactivate the colicin toxicity and maintain secretion, we removed 1 of the 3 predicted transmembrane regions within the colicin domain. The resulting VasX^{ΔC16} mutant was indeed inactivated and secreted, and in contrast to domain deletion this mutation has little effect on T6SS assembly (Fig. 2C). Considering the wide distribution of VasX-like effectors, this demonstration of inactivating colicin-like effectors with minimal structural effect would be informative to study other VasX homologs.

Not all species require effectors for T6SS assembly, nor do all effectors contribute to assembly equally. Indeed, combinatorial effector deletions in *A. tumefaciens* and *A. baylyi* have little effect on T6SS secretion (33, 34). This might result from differences in effector secretion routes since there are multiple routes of delivery for T6SS in different organisms (41). Both TseL and VasX rely on the VgrG spike as carrier for delivery, while other effectors are carried inside the Hcp tubule for delivery, as exemplified by the Tse1–4 effectors in *P. aeruginosa* (50). Considering the essential role of VgrG in T6SS assembly (18, 44), it is conceivable that VgrG-interacting effectors are more likely to modulate assembly. Of the 3 antibacterial effectors TseL, VasX, and VgrG3, lacking both VasX and VgrG3 abolishes T6SS secretion while TseL seems to contribute but is not critical for T6SS assembly (9). In addition, while VasX or VgrG3 alone showed strong *E. coli* killing activities, TseL alone did not (Figs. 1B and 3E).

Since VasX homologs can be found in a variety of species, the requirement for VasX or VgrG3 in T6SS assembly may represent a conserved resource-saving strategy to ensure the most effective toxins are delivered. This resembles an onboard checking strategy distinct from known mechanisms that conserve resources through transcriptional regulation of T6SS genes (51, 52) or through secretion of negative regulators (53, 54). The latter refers to the type III secretion system and the

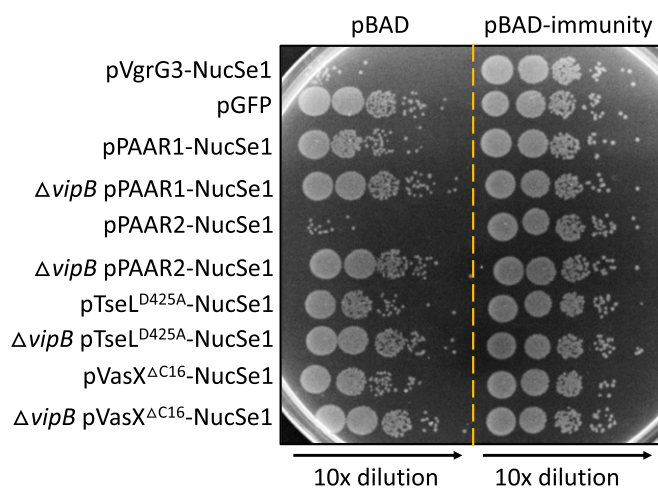


Fig. 5. Cytosol-to-cytosol delivery of chimeric cargo by the triple effector-inactive T6SS. Prey and killer cells are derivatives of the same parental *tseL*^{D425A}/*vasX*^{ΔC16}/*vgrG3*^{D842A} mutant except for carrying different plasmids or the T6SS null $\Delta vipB$ deletion as indicated. Survival of prey was compared by serial dilutions. Delivery of chimeric NucSe1 proteins would reduce the survival of prey with the empty vector but not the ones expressing the NucSe1-specific immunity protein. See *SI Appendix, Fig. S4* for comparison with statistics.

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