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Chimeric adaptor proteins translocate diverse type VI secretion system effectors in *Vibrio cholerae*

Daniel Unterweger, Benjamin Kostiuk, Rina Ötjengerdes, Ashley Wilton, Laura Diaz-Satizabal & Stefan Pukatzki*

Abstract

Vibrio cholerae is a diverse species of Gram-negative bacteria, commonly found in the aquatic environment and the causative agent of the potentially deadly disease cholera. These bacteria employ a type VI secretion system (T6SS) when they encounter prokaryotic and eukaryotic competitors. This contractile puncturing device translocates a set of effector proteins into neighboring cells. Translocated effectors are toxic unless the targeted cell produces immunity proteins that bind and deactivate incoming effectors. Comparison of multiple *V. cholerae* strains indicates that effectors are encoded in T6SS effector modules on mobile genetic elements. We identified a diverse group of chimeric T6SS adaptor proteins required for the translocation of diverse effectors encoded in modules. An example for a T6SS effector that requires T6SS adaptor protein 1 (Tap-1) is TseL found in pandemic *V. cholerae* O1 serogroup strains and other clinical isolates. We propose a model in which Tap-1 is required for loading TseL onto the secretion apparatus. After T6SS-mediated TseL export is completed, Tap-1 is retained in the bacterial cell to load other T6SS machines.

Introduction

Vibrio cholerae is a Gram-negative bacterium commonly found in the aquatic environment. The species *V. cholerae* is diverse, with over 200 described different serogroups. Only the O1 serogroup strains cause pandemic cholera, a potentially deadly disease characterized by severe watery diarrhea (Harris *et al*, 2012).

In the environment and in the human host, *V. cholerae* encounters competitors ranging from eukaryotic cells to prokaryotes of the same or other species. One mechanism of competition employed by *V. cholerae* requires direct contact and the type VI

secretion system (T6SS) (MacIntyre *et al*, 2010; Unterweger *et al*, 2012). Genes of the T6SS are encoded in one large cluster and two auxiliary gene clusters (Pukatzki *et al*, 2006). This secretion system forms a contractile puncturing device with homology to the tail-spike complex of the T4 bacteriophage inside the bacterial cell (Leiman *et al*, 2009). Contraction of the device's outer sheath ejects an inner tube from one bacterium into a neighboring cell (Basler *et al*, 2012). In the current model for this device, diverse effector proteins are localized to the conserved tip of the inner tube formed of Hcp and are ejected together with the Hcp tube (Ho *et al*, 2014; Russell *et al*, 2014; Zoued *et al*, 2014). Upon delivery of the effector proteins into the neighboring cell, the effectors have toxic effects unless inhibited by immunity proteins (Brooks *et al*, 2013; Dong *et al*, 2013). These immunity proteins protect cells from a T6SS-mediated attack by binding and deactivating T6SS effectors (Brooks *et al*, 2013; Zhang *et al*, 2014).

Vibrio cholerae uses the T6SS effectors VgrG-1, VgrG-3, TseL, and VasX (Brooks *et al*, 2013; Dong *et al*, 2013; Miyata *et al*, 2013) among others (Shneider *et al*, 2013; Altindis *et al*, 2015). These effectors are encoded by pandemic *V. cholerae* strains and also by the O37 serogroup strain V52, which has an active T6SS under laboratory conditions (Pukatzki *et al*, 2006; Unterweger *et al*, 2014). V52 is employed in this study as a representative strain that relies on this effector set. VgrG proteins are important for their structural role as part of a trimeric complex at the distal end of the Hcp tube (Leiman *et al*, 2009). VgrG-1 and VgrG-3 belong to a group of proteins with unique enzymatic C-terminal extensions attached to a core domain. Based on the covalent linkage of the C-terminal effector domain to the VgrG core domain, they are referred to as class 1 effectors (Shneider *et al*, 2013). The C-terminal domain of VgrG-1 contains an actin cross-linking domain that is toxic to phagocytic eukaryotic cells, such as murine macrophages (Sheahan *et al*, 2004; Pukatzki *et al*, 2007; Ma *et al*, 2009). The C-terminal domain of VgrG-3 contains a peptidoglycan-binding domain and confers toxicity toward other bacteria (Brooks *et al*, 2013; Dong *et al*, 2013). The genes encoding VgrG proteins are located upstream of TseL- and VasX-encoding genes. TseL and VasX are proposed to non-covalently bind surface features of VgrG proteins and are thus referred to as class 2 or cargo effectors (Shneider *et al*, 2013). VasX is a pore-forming effector that, by interfering with the

Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, AB, Canada
*Corresponding author. Tel: +1 780 492 0904; E-mail: spukatzki@ualberta.ca

inner membrane potential, lyses prokaryotic competitors (Miyata *et al*, 2011, 2013). TseL is a phospholipase (Dong *et al*, 2013; Russell *et al*, 2013) that degrades membranes of prokaryotic prey. The anti-prokaryotic effectors VgrG-3, VasX, and TseL are inhibited by their cognate immunity proteins TsiV3, TsiV2, and TsiV1, respectively.

Anti-prokaryotic T6SS effector and immunity proteins are encoded in T6SS effector modules that are likely mobile elements exchanged among *V. cholerae* strains (Unterweger *et al*, 2014). One effector module is found in each of the T6SS auxiliary gene clusters 1 and 2, and in the large gene cluster. The effector module in each gene cluster can differ from strain to strain. The gene clusters are distributed over both chromosomes of *V. cholerae* and also contain genes encoding additional T6SS proteins. Different strains encode different sets of effector modules, yet outside these modules, retain conserved genes of the T6SS, including genes encoding structural and regulatory components and an ATPase. The effector module set of any given *V. cholerae* strain determines the strain's T6SS effector repertoire and controls its interaction with other bacteria. For example, bacteria of two *V. cholerae* strains with different module sets kill each other in a T6SS-dependent manner (incompatible strains) because they carry different immunity proteins in their effector module sets (Unterweger *et al*, 2014). In contrast, two strains with the same module set do not die when attacking each other, but coexist (compatible strains) because they carry immunity proteins to the same effectors (Unterweger *et al*, 2014). We refer to this phenomenon as the "compatibility rule".

We observed that pandemic strains of *V. cholerae* harbor the same T6SS effector module set (Unterweger *et al*, 2014). Phylogenetic analysis of pandemic and non-pandemic *V. cholerae* strains and the effector module sets they harbor provide multiple signs of horizontal gene transfer of T6SS effector modules between distantly related strains (Unterweger *et al*, 2014). The power of these effectors depends on the environment the bacteria compete in. For example, the potent effector VasX creates pores in the membranes of prokaryotic competitors that only kill the cell in an environment of low osmolarity (Miyata *et al*, 2013). This environment is apparently not provided in the infant rabbit model system, in which T6SS-mediated killing depends on VgrG-3 but not VasX (Fu *et al*, 2013).

One class of diverse T6SS effectors is known to contain a conserved PAAR domain for recognition as T6SS substrates (Shneider *et al*, 2013). In contrast, effectors encoded in any of the three modules do not share a conserved domain, suggesting an alternative, PAAR-independent translocation mechanism. Here, we demonstrate that translocation of the effector TseL (encoded in auxiliary cluster 1) depends on VgrG-1, and a previously uncharacterized protein that we name the type VI secretion system adaptor protein 1 (Tap-1). The genes *vgrG-1* and *tap-1* are located immediately upstream of *tseL*. Tap-1 is a chimeric adaptor protein with conserved core region and varying N- and C-terminal segments. *Vibrio cholerae* strains encode a diverse set of chimeric Tap-1 proteins to enable secretion of various effectors in strain backgrounds with varying VgrG-1 proteins. Our analysis suggests that the chimeric adaptor proteins result from diversifying selection and recombination at a highly conserved site within *tap-1*.

Results

T6SS gene clusters encode proteins of the DUF4123 superfamily

The anti-prokaryotic T6SS effector TseL is encoded in the auxiliary cluster 1 of pandemic *V. cholerae* O1 serogroup strains and other clinical isolates such as the O37 serogroup strain V52 (Dong *et al*, 2013). We set out to elucidate the mechanism by which the T6SS effector TseL is translocated into neighboring cells. We took a candidate approach to decipher the molecular events that lead to TseL translocation. First, we evaluated the contribution of the uncharacterized ~33-kDa protein Tap-1, encoded immediately upstream of *tseL* (Fig 1A). BLAST (Altschul *et al*, 1990) analysis revealed that Tap-1 belongs to a superfamily of proteins with the domain of unknown function, DUF4123 (Fig 1B). This domain is about 120 amino acids in length and contains multiple conserved motifs: YLLLD, SPYxxLVxL, HLRxLLxV, and LFRFYDPxVL (Fig EV1A). DUF4123-containing proteins are found in over 100 bacterial species. Five representative species with DUF4123-encoding genes as part of their T6SS clusters are depicted in Fig 1C. DUF4123-containing proteins often contain additional conserved domains, including Fha, ZapA, and PWWP domains, all of which are involved in protein–protein interactions (Fig EV1B). Genes encoding the DUF4123 superfamily proteins are commonly found downstream of *vgrG* or putative effector-encoding genes in predicted T6SS gene clusters (Fig 1C). Some species encode multiple proteins of the DUF4123 superfamily in their genomes. For example, in addition to Tap-1, *V. cholerae* strain N16961 encodes VasW (VCA0019) (Fig 1C). We previously demonstrated that VasW is necessary for the secretion of and killing mediated by VasX, the pore-forming effector encoded directly downstream of *vasW* (Miyata *et al*, 2013). Even though Tap-1 and VasW belong to the same superfamily, the two proteins share < 20% identity in their amino acid sequences (Fig EV1C).

In summary, *tap-1* encodes a protein of the DUF4123 superfamily that is found in T6SS gene clusters of many bacterial species. Its conserved nature and physical proximity to T6SS effectors suggests a conserved role for Tap-1 in T6SS function.

Tap-1 is required for TseL translocation

Tap-1 is encoded upstream of *tseL*. Based on their close proximity, we investigated whether Tap-1 is required for TseL secretion and subsequent TseL-mediated killing. To determine the secretion requirements for TseL, V52 and an isogenic *tap-1* mutant were maintained in LB broth until they reached the mid-logarithmic phase of growth. TseL was found in the pellet and supernatant from V52, but was absent from the supernatant of the V52 mutant lacking *tap-1* (Fig 2A). *In trans* complementation of the *tap-1* null mutation restored detection of TseL in the supernatant. DnaK—a cytoplasmic protein used as a lysis control—was found in the pellet but not supernatant. We conclude that Tap-1 is required for T6SS-mediated translocation of TseL. To determine whether the secretion defect of the *tap-1* mutant is specific for TseL or affects T6SS-mediated secretion universally, we analyzed Hcp secretion in V52Δ*tap-1,* as Hcp secretion is the hallmark of a functional secretion system. We observed that Hcp is still secreted in the absence of Tap-1 (Fig 2A), suggesting that Tap-1 is necessary for the secretion
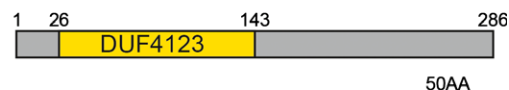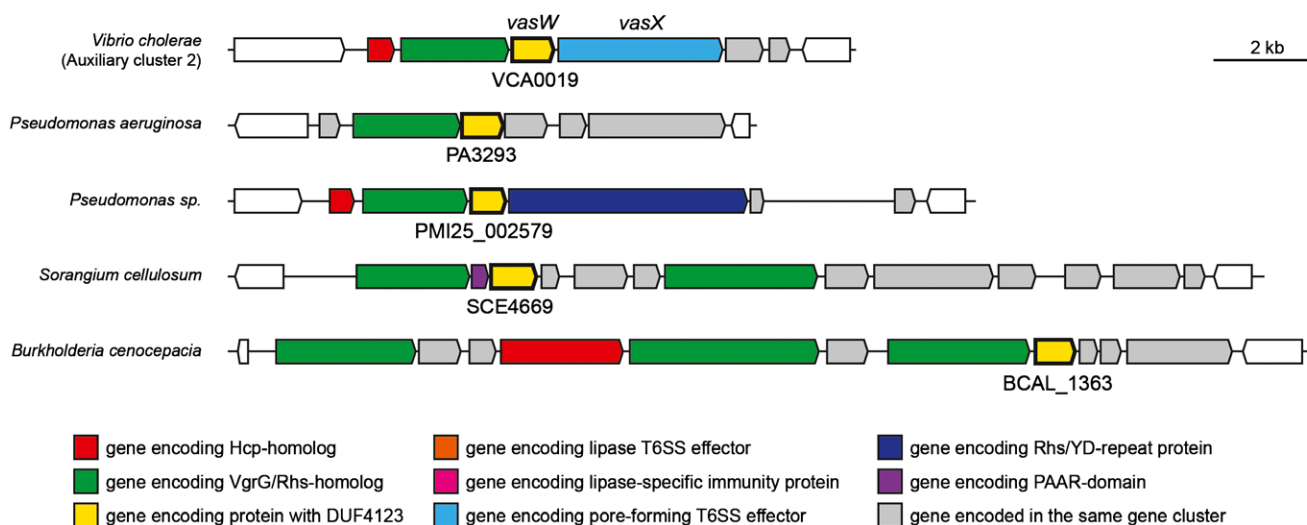
Figure 1. Tap-1 belongs to the superfamily of DUF4123 proteins.

A   Graphical depiction of the T6SS auxiliary gene cluster 1 of *Vibrio cholerae* strain N16961. Gene names are indicated above each gene. *Tap-1* (yellow) is outlined in bold, and its locus tag is indicated below the gene.
B   Graphical depiction of Tap-1 indicating the domain with homology to DUF4123.
C   Graphical depiction of DUF4123-containing gene clusters drawn to scale. The bacterial species encoding the genes are indicated on the left. All genes encoding proteins of the DUF4123 superfamily (yellow) are outlined in bold and labeled with their locus tag.

Data information: A legend for color coding is shown below (C). Legend indicates function predicted by BLAST. Genes of unknown function are colored in gray. Genes outside the operons are colored in white.

of TseL but not for other T6SS proteins. Tap-1 was not detected in culture supernatants by us and others, suggesting that Tap-1 is retained in TseL-secreting cells (Appendix Fig S1 and Altindis *et al*, 2015).

To determine whether the inability of a *tap-1* mutant to secrete TseL prevents killing of other prokaryotic cells in a TseL-dependent manner, we performed a killing assay in which V52 or V52Δ*tap-1* (predator) was mixed with C6706 or a C6706 mutant lacking the cognate immunity gene *tsiV1* (prey). Under these conditions, C6706 represses its T6SS while maintaining the expression of immunity genes (Miyata *et al*, 2013). The C6706Δ*tsiV1* mutant allowed us to analyze TseL-mediated killing because TsiV1 deactivates TseL in the bacterium under attack (here C6706) (Dong *et al*, 2013; Unterweger *et al*, 2014). Predator (V52) and prey (C6706) were mixed and plated on nutrient agar plates. After 4 h at 37°C, surviving V52 and C6706 were enumerated. The lack of *tap-1* abolished TseL-mediated killing by V52, comparable to a mutant lacking *tseL* (Fig 2B). Complementation with episomal *tap-1* restored TseL-mediated killing. Expression of *tap-1 in trans* in V52 had no effect on the survival of wild-type C6706 but killed the mutant lacking *tsiV1*, indicating that Tap-1 acts on TseL. These results show that TseL-mediated killing depends on Tap-1.

To test whether Tap-1 is required for the secretion of effectors in addition to TseL, we determined whether Tap-1 also controls VasX- and VgrG-3-mediated killing. As indicator strains for

VasX- and VgrG-3-mediated killing, we used C6706 mutant prey lacking the cognate immunity gene *tsiV2* and *tsiV3*, respectively. The absence of *tap-1* did not abolish killing mediated by VasX or VgrG-3 (Fig 2C). These results show that Tap-1 is specific for the effector TseL. A similar observation was made for VasW, the second protein of the DUF4123 superfamily in *V. cholerae*. VasW is only required for the effector VasX, encoded downstream of *vasW*, but is dispensable for TseL translocation (Appendix Fig S2). Similarly, a T6SS adaptor with a DUF1795 domain in *Serratia marcescens* also appeared to be specific for a single effector (Diniz & Coulthurst, 2015).

Taken together, these data indicate that Tap-1 is required for the secretion of TseL, allowing *V. cholerae* to engage in TseL-mediated killing.

VgrG-1 is necessary for secretion of and killing by TseL

The observation that Tap-1 is dedicated to TseL translocation encouraged us to probe other proteins encoded in auxiliary cluster 1 that act in concert with Tap-1. VgrG-1 is encoded immediately upstream of *tap-1* (Fig 1A). VgrG-1 in V52 is a structural component of the secretion system and is the carrier of an effector domain with actin cross-linking activity (Sheahan *et al*, 2004; Pukatzki *et al*, 2007; Ma *et al*, 2009). To determine whether VgrG-1 has a dual function (actin cross-linking and TseL translocation), we performed
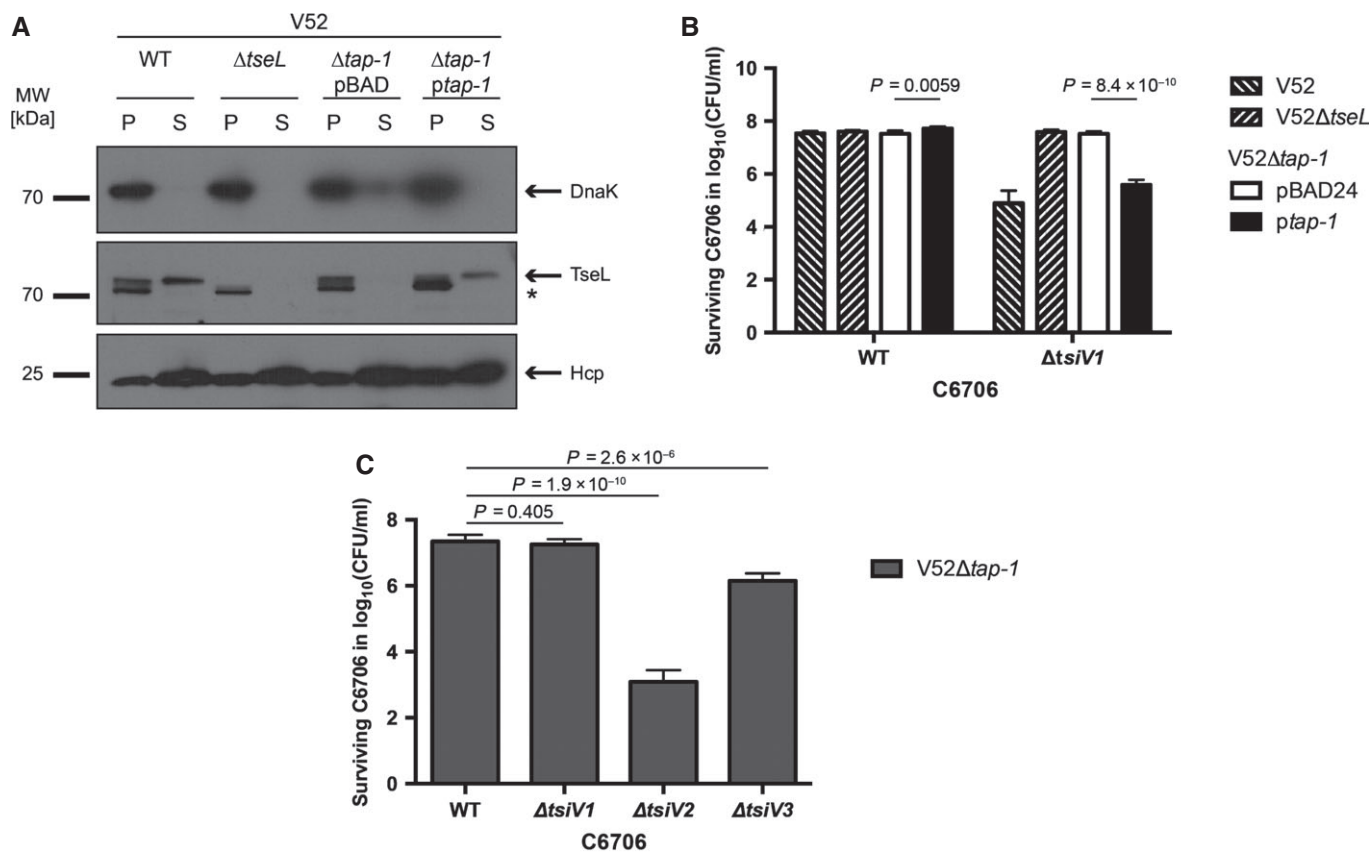
Figure 2. Tap-1 is required for TseL-mediated killing.

A   Tap-1 is required for the secretion of TseL. Pellet (P) and supernatant (S) of bacterial cultures were analyzed by SDS–PAGE. The result of immunoblotting with antisera against TseL and Hcp and purified antibodies against DnaK is shown. The asterisk marks an unspecific band, detected by the antiserum against TseL, present in the pre-immunized serum. One representative experiment of three independent experiments is shown.

B   Tap-1 is required for TseL-mediated killing. Wild-type C6706 or C6706 lacking *tsiV1* were exposed to the indicated V52 mutants in a killing assay. The *y*-axis indicates the number of surviving C6706.

C   Tap-1 is required for TseL function. Killing assays in which V52Δ*tap-1* was mixed with wild-type C6706 or mutants lacking *tsiV1*, *tsiV2*, or *tsiV3*. The *y*-axis indicates surviving C6706.

Data information: In (B, C), the arithmetic mean $\pm$ SD of three independent experiments each performed in duplicate is shown. *P*-values of a two-tailed, unpaired Student's *t*-test are indicated.
Source data are available online for this figure.

Western blot analysis on pellet and supernatant fractions of V52 and a *vgrG-1* mutant. As reported previously (Pukatzki *et al*, 2007), deletion of *vgrG-1* lowers the amounts of Hcp secreted by cells (Fig 3A). However, TseL secretion was abolished. Providing the *vgrG-1* mutant with episomal *VgrG-1* restored TseL secretion (Fig 3A). These observations indicate that VgrG-1 is required for the secretion of TseL.

Next, we used the killing assay to determine whether TseL-mediated killing occurred in the absence of VgrG-1. Parental V52 and mutants lacking *tseL* or *vgrG-1* were mixed with C6706 or C6706Δ*tsiV1*. A V52 mutant lacking *vgrG-1* lost its ability for TseL-mediated killing unless provided with episomal VgrG-1 (Fig 3B). These results show that *vgrG-1* is necessary for TseL-mediated killing.

To determine whether VgrG-1 is required for the translocation of VasX and VgrG-3 in addition to TseL, we performed killing assays using C6706 mutants lacking TsiV2 or TsiV3, the cognate immunity proteins for VasX and VgrG-3, respectively. TseL-mediated killing was abolished in the absence of *vgrG-1*, while VasX- and

VgrG-3-mediated killing still occurred (at reduced rates) in the absence of *vgrG-1* (Fig 3C).

While VgrG-1 and VgrG-3 differ in their C-termini, they share common core domains that could attract cargo effectors. To test whether VgrG-3 is necessary for TseL function, similar to VgrG-1, we determined the requirement of VgrG-3 for TseL secretion and TseL-mediated killing (Fig EV2). A *vgrG-1*-deficient mutant was unable to secrete TseL, while secretion of TseL was still observed in a mutant lacking *vgrG-3* (Fig EV2A). In the absence of *vgrG-3*, VgrG-3-mediated killing was abolished, but TseL-mediated killing was still observed (Fig EV2B and C). Even though VgrG-3 might be involved in the secretion of and killing by TseL, our results indicate that VgrG-3, unlike VgrG-1, is dispensable for TseL function.

To identify the domain of VgrG-1 required for TseL-mediated killing, we created VgrG-1 truncation mutants and tested their ability to restore TseL-mediated killing and Hcp secretion of a *tseL*-deletion strain (Fig EV3A, Appendix Fig S4). A truncated
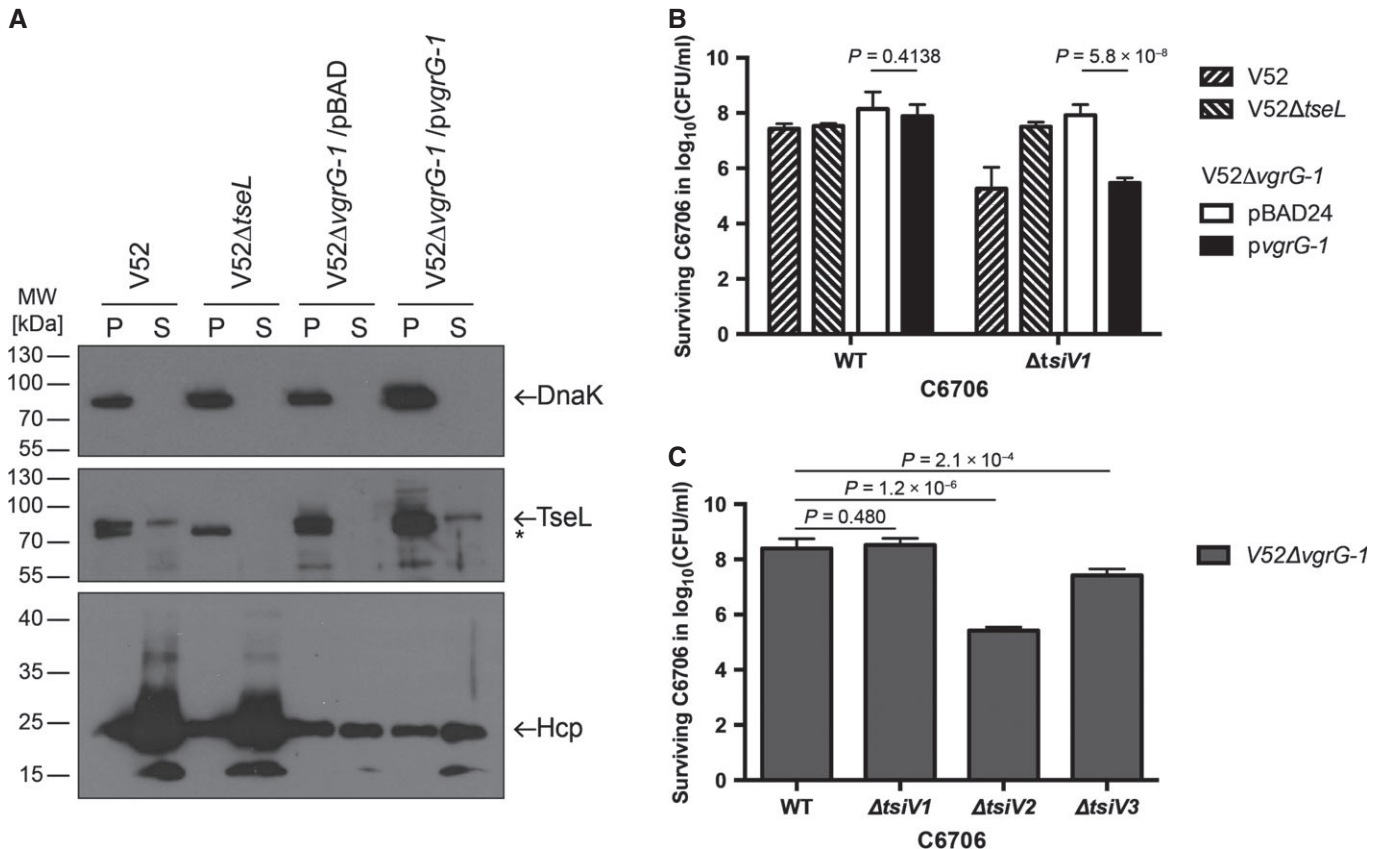
Figure 3. VgrG-1 is required for TseL-mediated killing.

A TseL secretion depends on VgrG-1. Western blot analysis of pellet (P) and supernatant (S) samples of the indicated strains. Samples were immunoblotted with antisera against TseL and Hcp and purified antibodies against DnaK. The unspecific band detected by rabbit serum is marked with an asterisk. One representative of three experiments is shown.

B VgrG-1 is required for TseL-mediated killing. Killing assay with wild-type V52 or indicated mutants and wild-type C6706 or a mutant lacking the immunity gene *tsiV1*. The *y*-axis indicates surviving C6706.

C VasX- and VgrG-3-mediated killing but not TseL-mediated killing occurs in the absence of VgrG-1. V52Δ*vgrG-1* was mixed in a killing assay with wild-type C6706 or mutants lacking immunity genes *tsiV1*, *tsiV2*, or *tsiV3* (specific for TseL, VasX, and VgrG-3, respectively). The *y*-axis indicates surviving C6706.

Data information: In (B, C), the mean ± SD of three independent experiments each performed in duplicate is shown. The *P*-values of a two-tailed, unpaired Student's *t*-test are indicated.

Source data are available online for this figure.

version of VgrG-1 missing the C-terminal actin cross-linking domain (ACD) (1–679) restored TseL-mediated killing and Hcp secretion similar to complementation with full-length VgrG-1 (Fig EV3B and C). An ACD-mutant of VgrG-1 lacking additionally amino acids 642–679 abolished TseL-mediated killing but maintained Hcp secretion. This 37-amino-acid-long sequence between position 642–679 connects the gp5-like domain with the actin cross-linking domain of VgrG-1 and is absent from VgrG-2 and VgrG-3 (Fig EV3D). We conclude that amino acids 642–679 are necessary for VgrG-1 to mediate killing by TseL. In summary, these results indicate that VgrG-1 is necessary for the secretion of and subsequent killing by TseL.

Tap-1 is required for the interaction between TseL and VgrG-1

The requirements for both VgrG-1 and Tap-1 to secrete TseL led us to investigate how these proteins contribute to the secretion process of TseL. VgrG-1 is secreted with other VgrG proteins

(Pukatzki *et al*, 2007) and PAAR-domain-containing proteins (Shneider *et al*, 2013) as part of a macromolecular complex at the tip of the secretion system. We hypothesized that TseL interacts with Tap-1 and VgrG-1 for recruitment to this complex. To test physical interactions among Tap-1, VgrG-1, and TseL, we performed immunoprecipitation experiments followed by Western blot analysis. First, we prepared lysates from V52Δ*tap-1* cells transformed with empty plasmid, or plasmid allowing expression of Tap-1 with a His-epitope (p*tap-1*::His) or FLAG-epitope (p*tap-1*::FLAG). FLAG-tagged Tap-1 was precipitated with anti-FLAG-M2 affinity beads. His-tagged Tap-1 is not expected to bind to the anti-FLAG-M2 affinity gel and allowed us to test for unspecific binding of TseL to the beads, or precipitation of inclusion bodies. Lysates and immunoprecipitates were subjected to SDS–PAGE and analyzed for the presence of TseL, Tap-1::His, and Tap-1::FLAG. TseL was found in the lysates of all three strains, but only in the immunoprecipitate generated with a FLAG epitope (Fig 4A). His-tagged Tap-1 was found in the
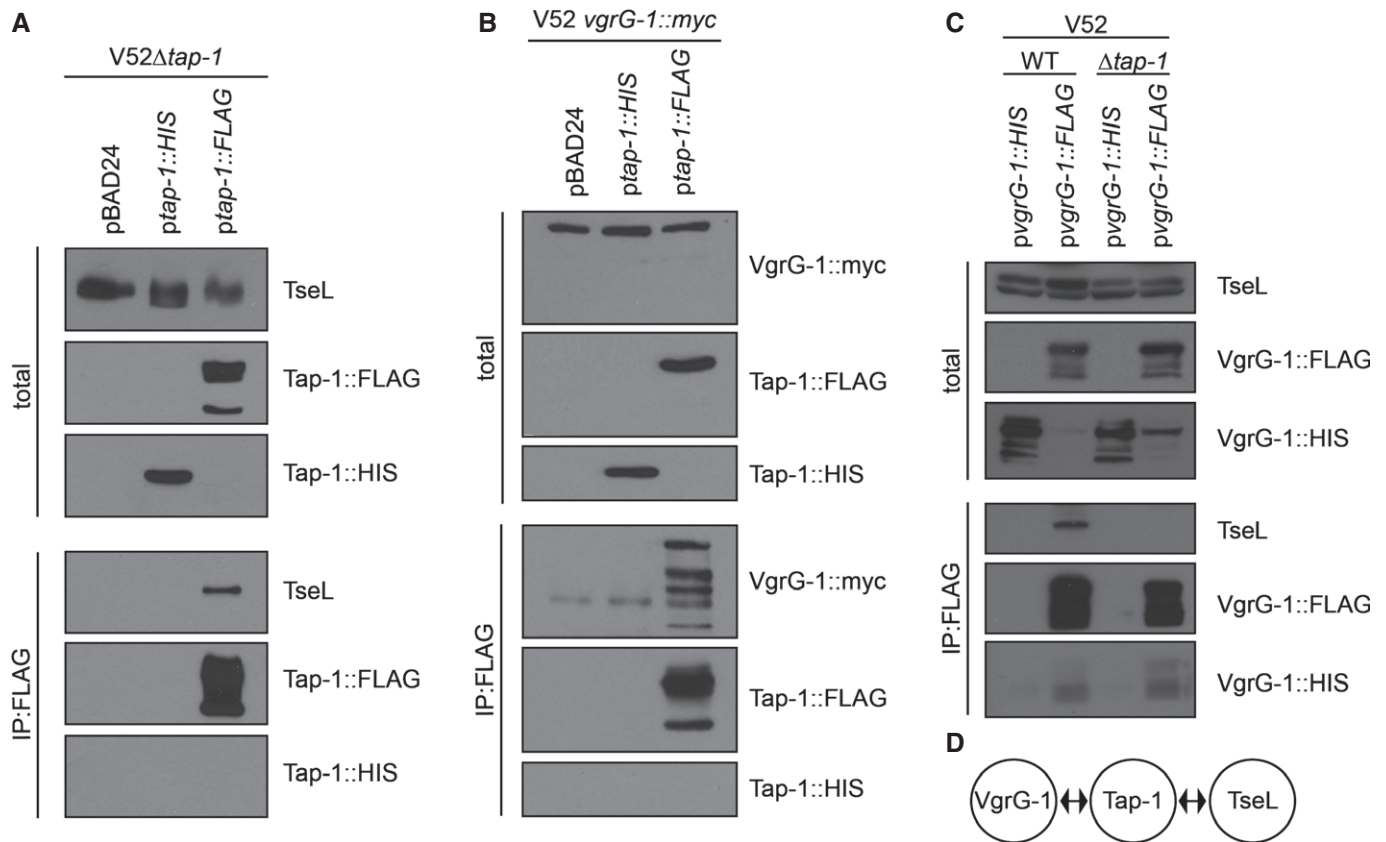
Figure 4. Tap-1 mediates interaction between TseL and VgrG-1.

A, B   Interactions between Tap-1 and TseL (A) or VgrG-1 (B). Shown are immunoblots of lysates (total) and immunoprecipitates with anti-FLAG affinity beads (IP:FLAG) of V52Δtap-1 (A) or V52 *ugrG-1::myc* (B) transformed with empty vector or a plasmid encoding His-tagged or FLAG-tagged Tap-1.

C   Interaction between TseL and VgrG-1. Shown are immunoblots of lysates (total) and immunoprecipitates with an anti-FLAG affinity beads (IP:FLAG) of V52 or V52Δtap-1 transformed with empty vector or a plasmid encoding HIS-tagged or FLAG-tagged VgrG-1.

D   Diagrammatic interpretation of (A–C). Arrows indicate interaction.

Source data are available online for this figure.

pellet but not in the supernatant. No band was detected with anti-TseL, anti-His, or anti-FLAG antibodies in precipitated lysates of V52Δtap-1 transformed with empty vector. This analysis established an interaction between Tap-1 and TseL.

To test whether Tap-1 also interacts with VgrG-1, we analyzed lysates of V52 with a chromosomal myc-tagged version of VgrG-1 that was transformed with either empty vector, p*tap-1*::His or p*tap-1*::FLAG. VgrG-1 was found in the lysates of all three strains but only in the immunoprecipitate with Tap-1::FLAG (Fig 4B). No band with the size of VgrG-1 was detected in the immunoprecipitate of lysates that contained His-tagged Tap-1 or untagged Tap-1. This suggests that VgrG-1 interacts with Tap-1.

As Tap-1 interacts with both VgrG-1 and TseL, we wanted to test the role of Tap-1 in the interaction between VgrG-1 and TseL. We tested lysates of V52 or V52Δtap-1 that express either His-tagged or FLAG-tagged VgrG-1. Anti-FLAG affinity beads were added to the lysates, pulled down, and analyzed with antibodies against TseL, FLAG, or His. TseL was detected in the immunoprecipitates with VgrG-1::FLAG only in wild-type V52, but not in the absence of Tap-1 (Fig 4C).

These results suggest that TseL has the ability to interact with Tap-1, which can interact with VgrG-1 (Fig 4D). TseL depends on

Tap-1 to form an interaction with VgrG-1, because we were unable to detect TseL in the immunoprecipitate of FLAG-tagged VgrG-1 from a lysate lacking Tap-1.

Based on our findings, we propose a model in which Tap-1 binds the effector and loads it onto the secretion apparatus.

Diversity of Tap-1 among *V. cholerae* strains

We previously showed that *V. cholerae* strains encode different sets of T6SS effector modules within their gene clusters (Unterweger et al, 2014). Auxiliary cluster 1 encodes one of two effectors depending on the strain (Unterweger et al, 2014). TseL is encoded in the T6SS gene clusters of strains such as the pandemic O1 serogroup strain N16961 and belongs to the A effector family. Effectors of the B family share < 30% amino acid sequence identity with TseL and are encoded in the T6SS gene clusters of strains such as the O39 serogroup strain AM-19226 or the O12 serogroup strain 1587. Having established a role for VgrG-1 and Tap-1 in the secretion of TseL (Figs 2 and 3), we hypothesized that VgrG-1 and Tap-1 have to be tailored for the translocation of A or B family effectors. First, we investigated the differences in VgrG-1 of different

strains. We compared the amino acid sequences of VgrGs encoded by *V. cholerae* strains used previously for our analysis of effector modules (Unterweger *et al*, 2014). Twenty-five of thirty-six strains encode a VgrG-1 protein that consists of the core domains and the C-terminal extension with actin cross-linking activity (Fig EV4). V52 and AM-19226 are examples of strains that contain a VgrG-1 with an actin cross-linking domain (ACD) (Fig 5A). 1587 is an example of a strain that contains a VgrG-1 without an ACD (Fig 5A). This analysis shows that VgrG-1 proteins of *V. cholerae* strains differ from each other by the presence or absence of a C-terminal extension.

Next, to analyze the diversity of Tap-1 proteins among *V. cholerae* strains, we aligned the amino acid sequences of

Tap-1 from the same 36 strains. Phylogenetic analysis showed that Tap-1 clusters into three clades (Fig 5B). An expanded analysis of all sequenced *V. cholerae* strains available in the NCBI database did not reveal additional Tap-1 alleles. Tap-1 from strains V52, AM-19226, and 1587 exemplifies clades 1, 2, and 3, respectively (Fig 5B). Alignment of three representative proteins from each cluster shows that they share multiple 15- to 33-residue-long motifs in the N-terminal segment (gray regions in Fig 5C). Motifs that make up domain DUF4123 are found in these highly conserved regions, except for the first motif, which is found in a non-conserved region in the polymorphic N-termini and varies at two amino acid positions between the analyzed sequences (Fig 5C). Differences in three representative sequences of the N-terminal segment (yellow
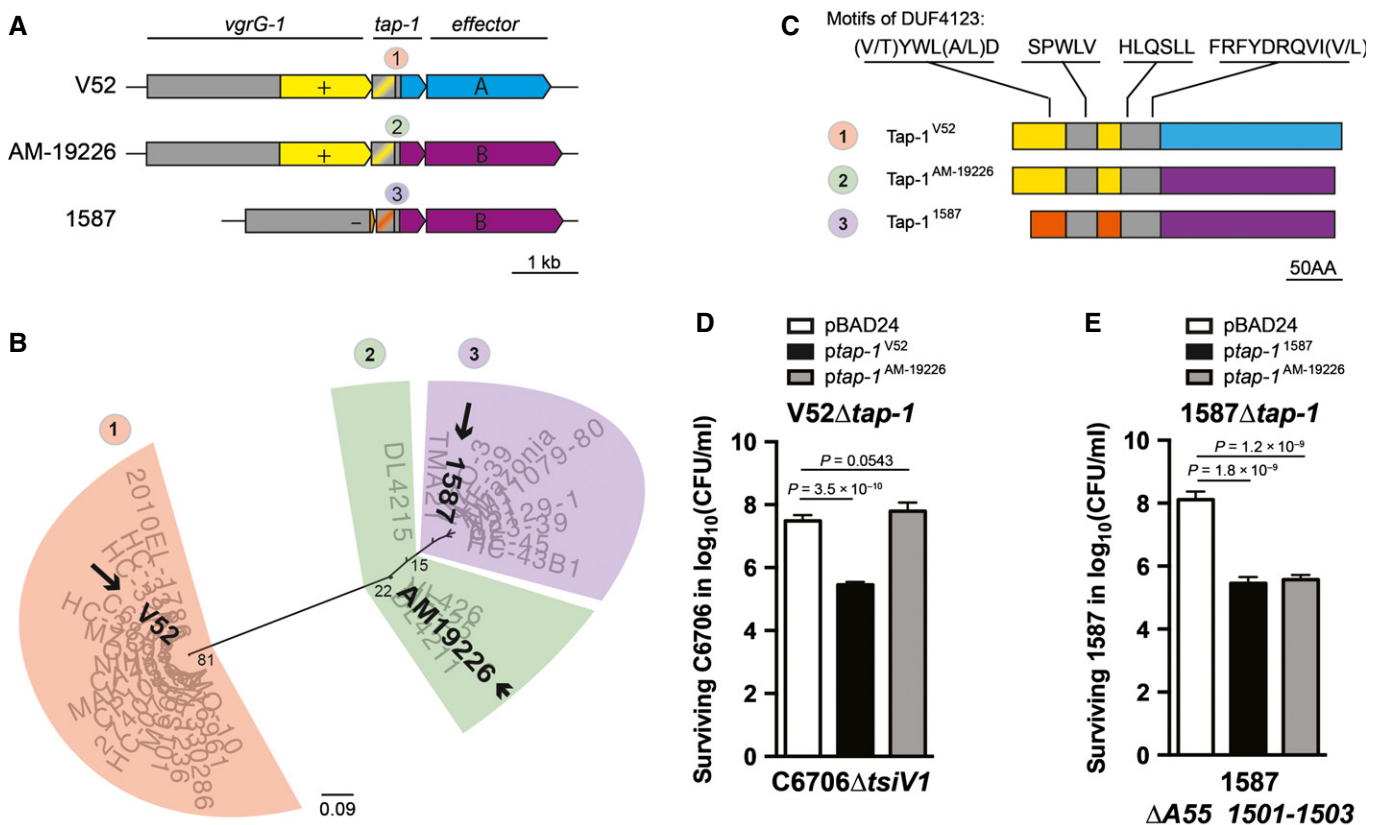

Figure 5. Diversity of Tap-1 among *V. cholerae* strains.

A   Cartoon of *ugrG-1*, *tap-1*, and the effector protein-encoding gene in the auxiliary cluster 1 of three indicated *V. cholerae* strains. Same color indicates more than 90% identity of the amino acid sequences encoded in the genes compared. Different colors indicate < 30% identity, and mixed color indicates 30–90% identity. The presence or absence of the ACD encoded in *ugrG-1* is indicated with + and −, respectively.

B   Diversity of Tap-1. Phylogenetic analysis of Tap-1 encoded in 36 *V. cholerae* strains. Analysis was performed using Raxml (Stamatakis, 2014). The names of Tap-1-harboring strains are indicated in the tree. Three clades are highlighted with different colors. Three representative sequences that cluster to different clades are highlighted with an arrow and used for analysis.

C   Cartoon of Tap-1 from the three different clades. Regions that share more than 80% identity in the three amino acid sequences are shown in the same color, and regions with < 45% identity are shown in different colors. The conserved sequences of the motifs of DUF4123 and their positions are indicated.

D   Specificity of the C-terminal Tap-1 segment. Killing assay in which the lack of *tap-1* in V52 is complemented by empty vector (control) or one of two different alleles of *tap-1*. The ability to kill in a TseL-dependent manner is indicated by the logarithm of surviving C6706Δ*tsiV1*.

E   Specificity of N-terminal segment of Tap-1. Killing assay in which the lack of *tap-1* in 1587 is complemented by empty vector (control) or one of two different alleles of *tap-1*. The ability to facilitate killing by the effector A55_1502 is indicated by the logarithm of surviving 1587ΔA55_1501-03.

Data information: In (D, E), the arithmetic mean ± SD of log-transformed data of three independent experiments, each performed in duplicate, is shown. *P*-values of a two-tailed, unpaired *t*-test are indicated.

Source data are available online for this figure.

and orange) and differences in the C-terminal segment (in blue and purple) are indicated in Fig 5C. Sequences in clusters 2 (e.g. Tap-1[AM19226]) and 3 (e.g. Tap-1[1587]) share highly similar C-terminal segments but varying N-terminal segments (Figs 5C and EV4C). Sequences in cluster 1 (e.g. Tap-1[V52]) contain highly similar N-terminal segments compared to sequences in cluster 2 (e.g. Tap-1[AM19226]) but contain distinct C-terminal segments that share < 30% amino acid identity (Fig 5C and Appendix Fig S3B). These analyses demonstrated that different yet distinct forms of Tap-1 are distributed among *V. cholerae* strains.

To test how differences in the N- or C-terminal segments of Tap-1 affect the function of Tap-1, we compared the adaptor proteins in pairwise combinations (see Fig EV5 for extended analysis of Tap-1 alleles in different strain backgrounds). The adaptors from V52 and AM-19226 share similar N-terminal segments but differ in their C-terminal segments (Appendix Fig S3B). To test whether differences in the N-terminal segment are tolerated, we compared the adaptor proteins from 1587 and AM-19226, which share common C-terminal segments. Both also share the conserved motives of the DUF4123 domain, but differ in the remaining sequence of the N-terminal segments. A 1587Δ*tap-1* mutant was provided with episomal *tap-1*[AM−192262] or *tap-1*[1587]. All Tap-1 alleles were expressed (Figs 5E and EV5B). Both adaptor proteins restored effector-mediated killing (Figs 5E and EV5A). This indicated that differences in the N-terminal segment are tolerated in the strain background tested. We were unable to test the Tap-1 alleles in the AM-19226 background because this strain did not engage in A55_1502-mediated killing. This could result from orphan immunity genes or from lack of expression of the effector module in AM-19226.

The adaptors from V52 and AM-19226 share similar N-terminal segments but differ in their C-terminal segments (Appendix Fig S3B). To determine the effector specificity of the C-terminal portion of Tap-1, V52Δ*tap-1* was provided with episomal *tap-1*[V52] or *tap-1*[AM−19226]. All Tap-1 alleles were expressed (Figs 5D and EV5B), but only Tap-1[V52] facilitated TseL-mediated killing of C6706Δ*tsiV1* at levels similar to wild type; Tap-1[AM−19226] did not support killing (Figs 5D and EV5A). This indicated that differences in the Tap-1 C-terminal segment are not tolerated.

Taken together, these data show that *V. cholerae* strains encode a variety of VgrG-1 and Tap-1 proteins. The VgrG-1 proteins differ from each other by the presence or absence of the enzymatically active C-terminal extension. The Tap-1 proteins differ from each other in their N- and C-terminal segments, with the C-terminal segments being specific for the T6SS effector. We found that the N-terminal segment of Tap-1 correlates with the presence or absence of a C-terminal extension of VgrG-1, and demonstrated that both N-terminal Tap-1 segments are operational in strains missing the ACD domain of VgrG-1.

N-terminus of Tap-1 evolved under diversifying selection

The N-terminal segment of Tap-1 correlates with the presence or absence of a C-terminal extension of VgrG-1 (Fig 5A and C). Despite the polymorphic nature of the N-terminal segment of Tap-1, the N-terminal segments of the 36 analyzed sequences still share an overall amino acid sequence identity of 64% (Fig 6A), indicating that they are homologs (Pearson *et al*, 2013).

We hypothesized that the two different N-terminal segments evolved from the same common ancestral segment. During this process, different amino acid sequences might have been selected for. Therefore, we analyzed retention of amino acid sequence versus nucleotide sequence by studying the effects of single nucleotide polymorphisms on the amino acid sequences of Tap-1 from all 36 analyzed strains (Fig 5). As an indicator for the number of non-synonymous mutations (change of amino acid sequence) and synonymous mutations (no change of amino acid sequence), we determined the ratio of percentage nucleotide sequence identity to amino acid sequence identity. We subdivided the 5′ end and N-terminal sequence into three regions, plus the middle region. The short, terminal 14-residue-long region of the N-terminal segment unique to classes 1 and 2 Tap-1 proteins (Fig 5) was excluded from this analysis, because this region would bias the analysis when comparing sequences of classes 1 and 2 to class 3 Tap-1 proteins lacking this region. Functional analysis demonstrated that this short region is not required for a functional Tap-1 (Appendix Fig S5), thus supporting our decision to exclude this fragment from our analysis. We observed that region 2 has a ratio of less than one (Fig 6B), demonstrating that the relative number of nucleotide substitutions is higher than the relative number of amino acid changes. This indicates selection pressure to retain the amino acid sequence but not the nucleotide sequence in region 2.

Regions 1 and 3 have a ratio greater than one (Fig 6B). Relatively few nucleotide changes cause a relatively high number of amino acid changes, indicating that these regions are selected to diversify.

Motifs of DUF4123 are found in regions 1 and 2 and the middle region, and are highly conserved on the amino acid level (Fig 6B). Even the WLxD motif found in the diverse region 1 is conserved, further highlighting the importance of this domain.

In summary, regions in the N-terminal segment of Tap-1 show signs of selection to conserve specific regions and to diversify others. The region in the middle of Tap-1 is highly conserved on both the nucleotide and amino acid levels, indicating its importance on both levels.

The middle region of Tap-1 is conserved on the nucleotide level

Genes encoding T6SS effector and immunity proteins differ in their GC content from the surrounding genes, indicating their acquisition and exchange among *V. cholerae* strains by horizontal gene transfer (Unterweger *et al*, 2014). For example, the strain VL426 is closely related to MZO-2 but encodes an effector other than *tseL* in its auxiliary cluster 1 (Unterweger *et al*, 2014). In contrast to the N-terminal segments of Tap-1 in our 36 analyzed sequences, the C-terminal segments of Tap-1 share < 30% overall amino acid identity (Fig 7A). This indicates a lack of common ancestry for the C-terminal segments and suggests different evolutionary origins. We hypothesized that *tap-1* contains a recombination site to create a diverse Tap-1 family.

Analysis of the nucleotide sequences of 36 *tap-1* genes revealed a highly conserved 99-bp region in the middle of the gene, with 92% identity (Fig 7A). Analysis of GC content shows that recombination likely happened at this site because the GC content differs by over 3% upstream and downstream of this site (Fig 7B). The 3′ end of *tap-1* differs in its GC content from the effector-encoding gene
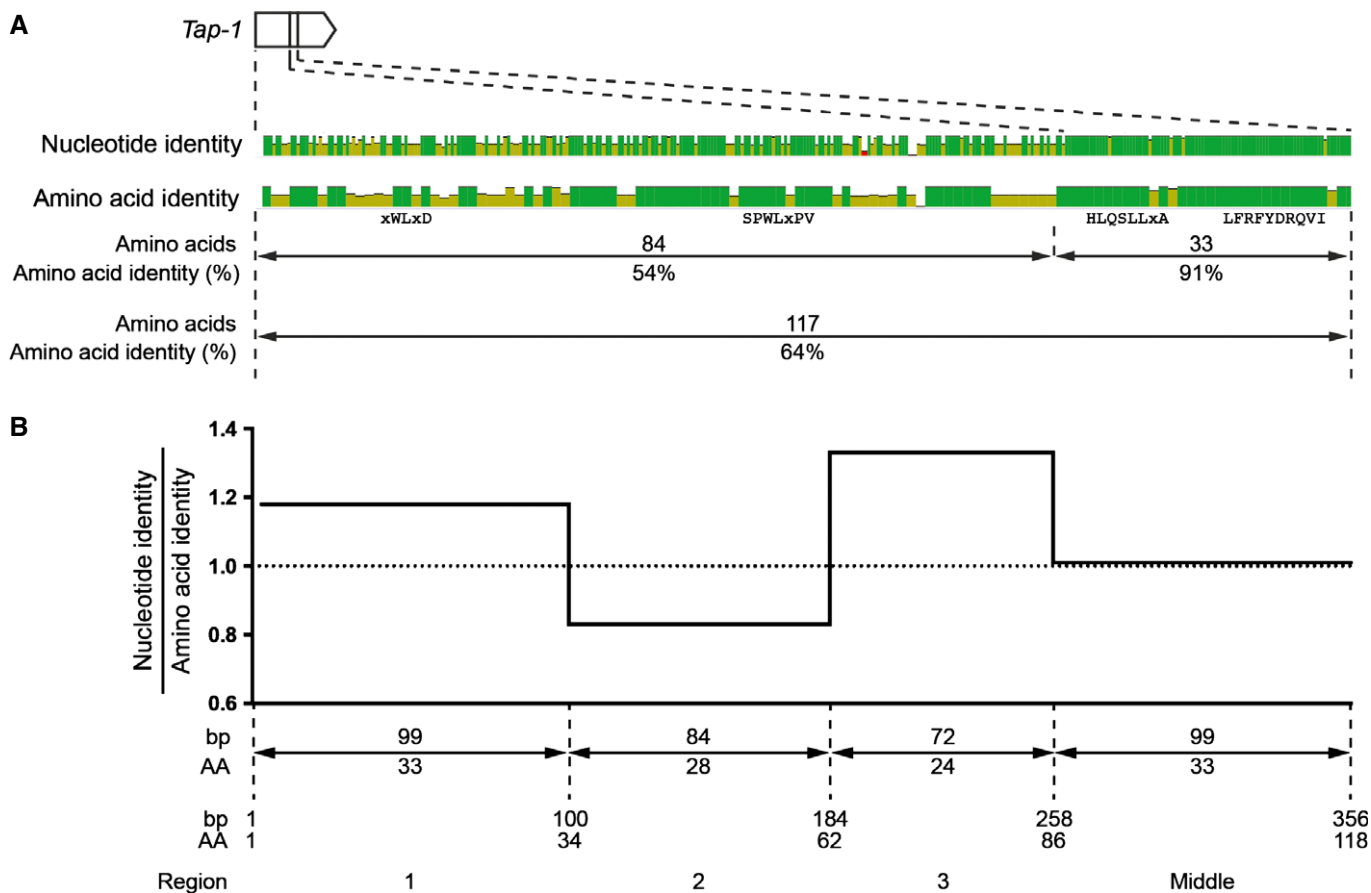
Figure 6. N-terminal domain of Tap-1 is under diversifying evolutionary selection.

A N-termini of Tap-1 share more than 30% overall amino acid sequence identity. The identity of the nucleotide and amino acid sequences across the indicated regions among *tap-1* of 36 *V. cholerae* strains (sliding window = 1) is shown. Green bars indicate 100% identity, yellow bars 30–99%, and red bars < 30%. The percent amino acid identity across the indicated region, with the length of amino acids indicated, is shown at the bottom of the figure. The first 14 amino acids of class 1 and 2 Tap-1 proteins were excluded from this analysis.

B The ratio of percentage nucleotide sequence change to percentage amino acid sequence change over the indicated region is shown in the graph.

downstream by only 0.6%, indicating that the 3′ end of *tap-1* plus the effector-encoding gene downstream could have been acquired and exchanged together between strains. Thus, we propose that the 3′ end of *tap-1* co-segregates with the effector-encoding gene downstream. Recombination in the core region would explain the lack of common ancestry among diverse C-terminal segments of Tap-1. Recombination within *tap-1* would maintain the open reading frame and give rise to a new protein with a different function than the proteins from which it derived its component pieces (Fig 5D); thus, we call Tap-1 a chimera.

Discussion

The competitive fitness of *V. cholerae* strains in regard to dealing with competitors, including other *V. cholerae* strains, is determined by the set of encoded T6SS effector modules (Unterweger *et al*, 2012, 2014). In this study, we took bioinformatics, genetic, and biochemical approaches to identify and characterize T6SS components required for the translocation of diverse effector proteins encoded in one of the three T6SS effector modules of auxiliary clus-

ter 1. Our analysis of the newly characterized adaptor protein Tap-1 revealed a chimeric composition with high diversity, which may have evolved through adaptation and recombination. The proposed mechanism of Tap-1-dependent effector translocation might especially be important for diverse, newly acquired effector proteins, which rely on a conserved T6SS for translocation. These findings advance our understanding of how VgrG proteins and the adaptor protein Tap-1 mediate T6SS effector translocation. Considering the numerous types of bacteria with a T6SS secretion system and the ubiquity of proteins belonging to the DUF4123 superfamily, the implications of our findings are likely to reach well beyond *V. cholerae*.

We propose a model in which the C-terminal segment of Tap-1 binds the cognate, downstream-encoded effector TseL. The N-terminal segment of Tap-1 then binds VgrG-1, which recruits the TseL effector to the tip of the T6SS central Hcp tube in preparation for translocation into a neighboring cell. As we do not detect Tap-1 in the supernatant of *V. cholerae* cells engaged in constitutive T6SS-mediated translocation of TseL, we hypothesize that Tap-1 hands over TseL to VgrG-1 prior to the ejection of the effector-loaded central Hcp tube. Such a mechanism would retain Tap-1 in the cytoplasm as shown in Appendix Fig S1, making
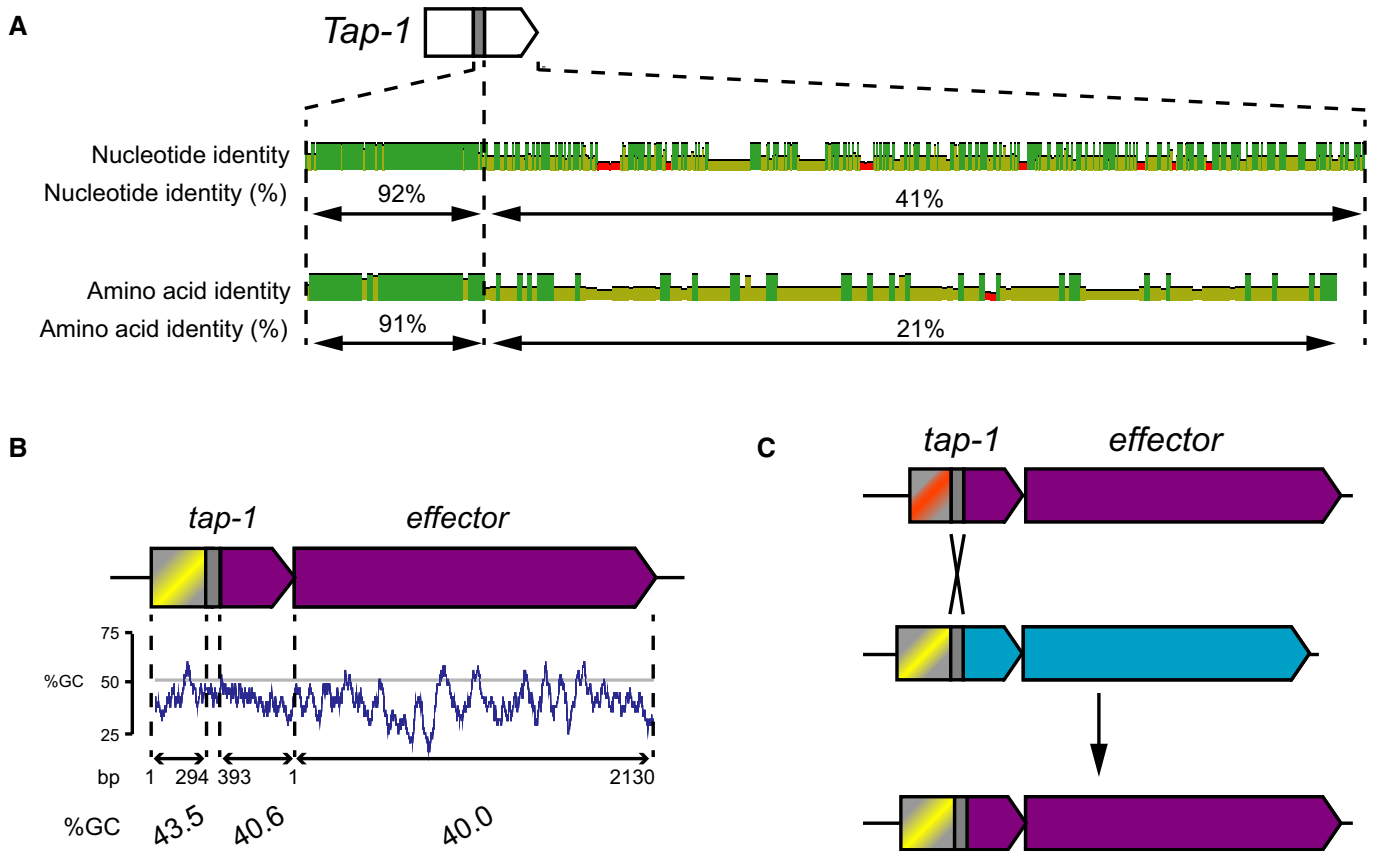
Figure 7. *Tap-1* encodes a putative recombination site.

A   Alignments of the nucleotide and amino acid sequences of the Tap-1 C-termini. Identity in 36 amino acid sequences of Tap-1 or nucleotide sequences of *tap-1* are shown (sliding window = 1). Green bars indicate 100% identity, yellow bars indicate 30–99%, and red bars indicate < 30%. Average percent identity is shown over the regions indicated with arrows.

B   Analysis of the GC content (sliding window = 50) of *tap-1* and the effector-encoding gene in *V. cholerae* strain 1587. Average percent GC in regions indicated with arrows is shown.

C   Model for the acquisition of a new 3′ end for *tap-1* and an effector-encoding gene as a result of the recombination site within *tap-1*, and an additional downstream recombination event (not shown).

Tap-1 available for loading of the next Hcp tube. A recent study by Whitney and colleagues on the T6SS of *Pseudomonas aeruginosa* and *Enterobacter cloacae* showed that VgrG proteins are also required for the translocation of PAAR-domain-containing effectors (Whitney *et al*, 2014; Shneider *et al*, 2013). We speculate that TseL interacts with VgrG at a different site than PAAR-domain effectors, because TseL is missing the PAAR domain. To which extent the herein described interactions are direct or depend on additional proteins needs to be further elucidated. A direct interaction between VgrG-3 and TseL has previously been proposed based on the detection of TseL in immunoprecipitates of VgrG-3 (Dong *et al*, 2013). Our results do not exclude such an interaction, but rather suggest that VgrG-1 is indispensable for TseL, whereas VgrG-3 is dispensable.

Our characterization of Tap-1 reveals that a protein of the DUF4123 superfamily is required for the interaction between a T6SS effector and its cognate VgrG protein, subsequent effector secretion, and effector-mediated killing. We made similar observations for another member of the DUF4123 superfamily, VasW (Appendix Fig S2 and Miyata *et al*, 2013). Genes encoding DUF4123 domains

are also found located immediately upstream of effector genes with a MIX (marker for type VI effectors) motif (Salomon *et al*, 2014), suggesting that one conserved role for DUF4123-containing proteins might be to assist in T6SS-mediated effector delivery. The function of Tap-1 is characteristic of a chaperone protein in terms of binding cargo effectors and delivering them to the secretion apparatus as described for chaperons in a variety of secretion systems, including the type III and type VII secretion system (Page & Parsot, 2002; Daleke *et al*, 2012). Such a chaperone function has been described for Hcp from *P. aeruginosa*, which harbors T6SS effectors smaller than 20 kDa (such as Tse2) inside the Hcp conduit prior to ejection (Silverman *et al*, 2013). In the absence of Hcp, Tse2 becomes unstable. Proteins of the DUF4123 superfamily might also stabilize their cognate effector, as a recent study by Ma and colleagues suggests. When the authors purified recombinant Tde1, an *Agrobacterium tumefaciens* T6SS effector, higher yields were obtained when co-expressed with a member of the DUF4123 protein superfamily (Ma *et al*, 2014). Tap-1 may also have a stabilizing function for TseL that we may have not detected due to the non-quantitative nature of luminol-based chemiluminescent

detection. In summary, these findings show that certain T6SS effectors require additional proteins for stability or secretion and that different types of effectors depend on different types of accessory proteins such as Tap-1 or Hcp.

Our analysis of VgrG-1 revealed that not all VgrG-1 proteins contain a C-terminal extension that confers anti-eukaryotic activity. The actin cross-linking domain (ACD) of VgrG-1 is an example of an effector domain that is missing in some strains, not replaced by another effector domain. The T6SS gene clusters of *V. cholerae* thus not only differ between strains in their composition of T6SS effectors but also in their total number of effectors. We observed that *V. cholerae* strains encode a diversity of Tap-1 proteins that differ in their N- and C-terminal segments. The N-terminal segment of Tap-1 of *V. cholerae* strains that contain VgrG-1 with an ACD differs from the N-terminal segment of Tap-1 of strains that contain VgrG-1 without an ACD. The C-terminal segments differ between Tap-1 of strains that differ in their anti-prokaryotic effector; thus, the C-terminal segment appears to display specificity for the cognate effector they encode. Of the four possible combinations of the two types of N-terminal and C-terminal segments, we found only three combinations in an exhaustive search of publically available genomic sequences for *V. cholerae* strains. The lack of a Tap-1 chimera with the N-terminal segment found in strains without the ACD and the C-terminal segment specific for TseL suggests a functional relationship between ACD and TseL.

Key to the chimeric structure of Tap-1 is the putative recombination site in the middle of *tap-1*. We speculate that the 3′ end of *tap-1* downstream of the recombination site co-segregates with the effector-encoding gene. We now include this 3′ end of *tap-1* in our definition of a T6SS effector module, in addition to genes encoding an effector protein and an immunity protein. We envision a crucial role for *tap-1* during the exchange of effector modules that determine compatibility. Our previous phylogenetic analysis of *V. cholerae* strains found that distantly related strains encode the same effector module set (Unterweger *et al*, 2014). This could be explained by exchange of effector modules through horizontal gene transfer and subsequent compatibility group switching. Despite this possibility of T6SS module exchange, pandemic strains all encode the same module set, AAA (Unterweger *et al*, 2014). The fact that pandemic strains have conserved T6SS effectors indicates selection for their module set. All herein analyzed pandemic strains also contain a VgrG-1 with an ACD. Thus, it appears that the ACD and the AAA module sets comprise the characteristic effector module set of pandemic strains in addition to the presence of cholera toxin and toxin co-regulated pilus.

T6SS-mediated competition interferes with cell–cell contact and thereby affects contact-dependent exchange of genetic elements. When *V. cholerae* inhabits chitin surfaces of copepods in environmental reservoirs, the T6SS and competence (the ability to take up extracellular DNA) are coupled (Borgeaud *et al*, 2015). Our "compatibility rule" predicts that a predator *V. cholerae* strain will take up the genomic DNA from incompatible prey that succumb to the T6SS-mediated attack and are lysed. Any double crossovers that include a complete T6SS cluster like the small, *tseL*-containing auxiliary cluster 1 will replace the predator cluster with the donor prey cluster. This prey cluster should be functional in predator, because the prey cluster provides the proper adaptor gene that operates with the new effector. However, if the predator carries a VgrG-1 with an actin cross-linking domain and the prey does not, then the recipient gains a new activity, but loses the ability to cross-link actin. We hypothesize that recombination in the *tap-1* core splices a new 3′ end to the existing 5′ end of *tap-1* on the chromosome, allowing the cell to use its resident T6SS to secrete newly acquired effectors, while retaining the ACD on VgrG-1. In consequence, the recipient predator bacterium would change its compatibility group, allowing the strain to kill bacteria with which it was previously compatible—including its parental kin. The ability to horizontally acquire genes from incompatible strains in the environment permits strains to diversify and to select for adaptive traits beneficial for persistence in the environment or the host.

Materials and Methods

Strains and culture conditions

Vibrio cholerae strains were grown in Luria Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at 37°C with shaking. As indicated, bacteria were grown in the presence of 100 μg/ml ampicillin, 100 μg/ml streptomycin, or 50 μg/ml rifampicin. Expression from pBAD24 was induced with 0.1% arabinose.

Molecular cloning

The expression vector pBAD24 (Guzman *et al*, 1995) was used to express genes *in trans*. T6SS genes were amplified using indicated primers (see Appendix), cloned into plasmid pJet (Thermo Scientific) and subsequently inserted into pBAD24. In-frame deletion mutants were created as described previously (Metcalf *et al*, 1996) using pWM91-based knockout constructs.

Killing assays

Killing assays were performed as described in MacIntyre *et al* (2010). Briefly, *V. cholerae* V52 and C6706 were mixed at a 10:1 ratio and incubated on a pre-dried LB agar plate. If applicable, bacteria were plated onto a LB agar plate supplemented with 0.1% arabinose to induce expression *in trans*. After a 4-h incubation at 37°C, bacteria were harvested, serially diluted and plated onto LB agar plates supplemented with selective antibiotics to enumerate surviving V52 or C6706. The next day, colony-forming units (CFUs) were counted.

Immunoprecipitation

Cell lysates were prepared as described in Dong *et al* (2013). Briefly, overnight cultures were diluted 1:100 and grown to a late mid-log optical density (OD) in the presence of the corresponding antibiotic, induced with 0.1% arabinose, and grown for another hour at 37°C shaking at 220 rpm. Cells were harvested by centrifuging for 10 min at 4,000× *g* and resuspended in TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6) supplemented with protease inhibitor (Roche) and 1 mg/ml lysozyme (Sigma). Cells were lysed by sonication and debris was removed by centrifugation at 10,000× *g* for 15 min (4°C). The supernatant was mixed with sample buffer and boiled for detecting proteins in crude extracts (total), or treated with

anti-FLAG affinity beads (Sigma) for 2 h at 4°C. Beads were washed, and precipitated protein eluted by boiling the beads with sample buffer.

Western blot analysis

Secretion of T6SS proteins was analyzed as described previously (Brooks *et al*, 2013; Dong *et al*, 2013). Bacterial cultures were grown to an OD of ~0.5. Cultures were grown for another hour in the presence of 0.1% arabinose to induce gene expression from pBAD24. Bacteria were pelleted and supernatants were filtered through 0.22-μm filters and concentrated via trichloroacetic acid (TCA) precipitation. Samples were loaded onto 10% acrylamide gels, subjected to SDS gel electrophoresis, and blotted onto nitrocellulose membranes. The following primary antibodies were used: rabbit anti-Hcp (1:500, Pukatzki *et al*, 2006), rabbit anti-TseL (1:5,000, Southern Alberta Cancer Institute, Calgary, Canada), mouse anti-DnaK [1:15,000, Enzo Life Sciences (ADI-SPA-880)], mouse anti-HIS [1:1,000, Santa Cruz (SC53073)]. Secondary antibodies conjugated to horseradish peroxidase were used: goat anti-rabbit [1:3,000, Santa Cruz (SC-2004)], goat anti-mouse [1:3,000, Santa Cruz (SC-2005)], and, to detect native mouse antibody after IP, anti-mouse [1:1,000, Abcam (ab131368)].

Bioinformatics

MUSCLE (Edgar, 2004) was used for all alignments of nucleotide and amino acid sequences.

The phylogenetic tree of 36 Tap-1 sequences was created using Raxml (Stamatakis, 2014) with the following settings: algorithm, rapid hill-climbing; protein model, blosum 62; bootstrap = 100. Protein–protein blastp (Altschul *et al*, 1990) search against the non-redundant protein sequences was performed using the amino acid sequence of VC1417 as the query.

The GC content of an indicated nucleotide sequence region and the percentage of identity among nucleotide or amino acid sequences were determined by the Geneious analysis tool (Kearse *et al*, 2012).

To determine the ratio of nucleotide identity to amino acid identity, the percentage identity of nucleotide and amino acid sequences among the indicated regions was determined, then the percentage identity of nucleotide sequences was divided by the percentage identity of amino acid sequences.

To determine the conserved motif of DUF4123, the 100 most diverse amino acid sequences were retrieved from the NCBI conserved domain database (Marchler-Bauer *et al*, 2013). Sequences were loaded into WebLogo 3 (Crooks *et al*, 2004) for graphical display of the alignment. The nine proteins representing the combination of the DUF4123 domain with various other domains were chosen after analysis with the NCBI conserved domain architecture retrieval tool (Geer *et al*, 2002) using the amino acid sequence of VCV52_1391 as a query. The nucleotide sequence of the gene encoding for DUF4123 superfamily proteins plus the surrounding region was downloaded from NCBI. Each annotated open reading frame was translated into the amino acid sequence and subjected to a BLAST search (Altschul *et al*, 1990) to look for homology to characterized proteins with known functions.

The model for the structure of VgrG-1 was created using Phyre2 (Kelley *et al*, 2015), intensive modeling mode.

Software

For data processing and the creation of figures, we used Microsoft Office 2010, GraphPad Prism 6, Adobe Cloud CC, and Geneious R8 (Kearse *et al*, 2012).

Statistical analysis

An unpaired, parametric *t*-test was used to determine statistical significance of differences between two indicated groups of data points. The logarithm of the CFU/ml was used as the basis for the analysis. Only data of equal variances were compared, controlled for by a non-significant *F*-test. *P*-values smaller than 0.05 were considered significant.

Author contributions
DU, BK, RÖ, AW, LD-S, and SP performed experiments. DU and SP designed experiments and interpreted results. DU, BK, and SP wrote the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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