**Vibrio cholerae** Requires the Type VI Secretion System Virulence Factor VasX To Kill *Dictyostelium discoideum*\(^\dagger\)

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The type VI secretion system (T6SS) is recognized as an important virulence mechanism in several Gram-negative pathogens. In *Vibrio cholerae*, the causative agent of the diarrheal disease cholera, a minimum of three gene clusters—one main cluster and two auxiliary clusters—are required to form a functional T6SS apparatus capable of conferring virulence toward eukaryotic and prokaryotic hosts. Despite an increasing understanding of the components that make up the T6SS apparatus, little is known about the regulation of these genes and the gene products delivered by this nanomachine. VasH is an important regulator of the *V. cholerae* T6SS. Here, we present evidence that VasH regulates the production of a newly identified protein, VasX, which in turn requires a functional T6SS for secretion. Deletion of vasX does not affect export or enzymatic function of the structural T6SS proteins Hcp and VgrG-1, suggesting that VasX is dispensable for the assembly of the physical translocon complex. VasX localizes to the bacterial membrane and interacts with membrane lipids. We present VasX as a novel virulence factor of the T6SS, as a *V. cholerae* mutant lacking vasX exhibits a phenotype of attenuated virulence toward *Dictyostelium discoideum*.

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Secreted proteins are those dependent on the structural apparatus to exit the cell. Finally, translocated proteins are effectors, which are secreted through the injectosome directly into host cells.

Although rapid progress in understanding the contribution of the structural components that make up the physical T6SS apparatus is being made, little is known about the regulation of the genes encoding them. A better understanding of T6SS gene regulation will allow us to determine when and where this virulence mechanism is engaged during V. cholerae’s complex life cycle and to assess whether and how the T6SS contributes to the diarrheal disease cholera. A vasH mutant is unable to export Hcp and displays a phenotype of attenuated virulence toward amoebae and mammalian macrophages (34), demonstrating a central role for VasH in T6SS-mediated virulence. In this study, we report the identification of a VasH-regulated, 121-kDa virulence factor designated VasX, whose T6SS-dependent transport contributes to the virulent behavior of V. cholerae.

MATERIALS AND METHODS

Strains and culture conditions. D. discoideum AX3 was grown in shaking liquid culture in HL5 medium (40) at 22°C. A V. cholerae V52 derivative strain lacking hlyA, hpaA, and recA was the background strain (34) for all T6SS deletion mutants and was used as the wild-type strain in all experiments. A vasX mutant and was used as the wild-type strain in all experiments.

In-frame deletion of T6SS genes

In-frame deletions and complementation. PCR products resulting from primer combinations A-KOvasX/B-KOvasX and A-vasH/B-vasH were used for cloning and mating purposes, respectively. All bacterial strains were grown in LB broth at 37°C. Murine RAW 264.7 mutants and was used as the wild-type strain in all experiments. 121-kDa virulence factor designated VasX, whose T6SS-de

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Cloning, expression, and purification of recombinant VasX. The full-length vasX gene was cloned from a Gateway entry vector (Harvard Institute of Proteomics clone identification no. VeCd00020120) into the pET-DEST42 expression vector in frame with a C-terminal V5 and 6xHis fusion for IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible high-level expression. The resulting plasmid, pET-DEST42-vasX, was transformed into E. coli BL21 Star (DE3) (Invitrogen), which encodes the T7 RNA polymerase required for expression from the T7 promoter. The E. coli BL21 Star (DE3)/pET-DEST42-vasX culture was incubated at 30°C to an OD_600 of ~0.5, induced with 0.1 mM IPTG, and harvested 4 h postinduction by centrifugation at 5,000 × g.

Primers vasX-pET28a-F (5'-GGTTGCTGATGATGAACTCCCAATCTGAGCTGC-3') and vasX-pET28a-R (5'-GGTGTGCTGATGAACTCGCTCAGGTGCGGCTC-3') were used to amplify the N-terminal 200 residues of VasX for cloning into the pET28a vector (Novagen) using the restriction sites Nhel and Xhol to place the fragment in frame with an N-terminal 6xHis tag. The construct, pET28as-vasX, was transformed into E. coli BL21 Star (DE3) and grown at 25°C to an OD_600 of ~0.9, induced by the addition of 10 μM IPTG for 16 to 20 h, and harvested at centrifugation of 5,000 × g.

Bacterial pellets were resuspended in 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 20 mM imidazole supplemented with 1 mM phenylmethylsulfonyl fluoride and lysed by three passes through a French pressure cell at 20,000 × g and filtered through 0.45-μm PVDF filter (Millipore). His-tagged proteins were purified using the AKTAadex Basic System (GE Healthcare, Piscataway, NJ) mounted with a HisTrap FF Crude (GE Healthcare) column using an elution gradient of 20 mM to 500 mM imidazole. The purified protein fractions were dialyzed against PBS (pH 7.4), and the concentration was determined by A_280.

**RESULTS**

Identification of VasX. In an attempt to identify secreted/exported *V. cholerae* proteins that require VasH for expression, we used a complemented vasH-null mutant of *V. cholerae* (V52ΔvasH) that carries the plasmid pBAD24-vasH with vasH under the control of an arabinose-inducible promoter. We compared SDS-PAGE protein profiles from culture supernatants in which bacteria were grown in the presence or absence of arabinose. Supernatants of strains grown in the presence of arabinose revealed multiple bands absent from supernatants of *V. cholerae* bacteria grown in the absence of arabinose (Fig. 1A). Given that V52ΔvasH alone does not secrete Hcp (34), restoration of Hcp secretion to wild-type levels upon vasH induction indicated proper complementation (Fig. 1A and data not shown). In addition to Hcp, an ~120-kDa band appeared when VasH expression was induced. The 100- to 140-kDa range from each lane was excised and subjected to in-gel tryptic digestion followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Mass spectrometry led to the identification of a protein with a molecular mass of 121 kDa in supernatants of VasH-producing bacteria that was not present in supernatants of bacteria lacking VasH. We named the 121-kDa protein VasX (VCA0020). In addition to VasX, other proteins present only in supernatants of vasH-induced bacteria were identified by mass spectrometry, and these proteins included VgrG-1, VgrG-3, and Hcp (Fig. 1B). Surprisingly, even though VgrG-2 is encoded directly upstream of vasH, it was not identified from culture supernatants when vasH was overexpressed. The VasX protein is encoded on the *V. cholerae* small chromosome immediately downstream of hcp-2, vgrG-2, and VCA0019.
mediated virulence. The NCBI denotes VasX as a hypothetical protein, and BLASTp analysis revealed that VasX homologs are present in Gram-negative bacteria such as *Pseudomonas syringae*, *Photobacterium damselae*, and *Aeromonas hydrophila*. The hypothetical gene VCA0019 is located between vgrG-2 and vasX and also belongs to this T6SS gene cluster, but its role in virulence remains to be determined.

The hidden Markov model homology search program HHpred (38) identified an N-terminal pleckstrin homology (PH) domain (E = 0.5; P = 5E−5) near the N terminus of VasX. Although the E values identified by HHpred are large, further protein structure prediction of the candidate PH domain (amino acids 73 to 166) in VasX by the Phyre server (5, 19) also indicated a canonical PH domain structure consisting of two β-sheets followed by an α-helix (21). Since PH domain-containing proteins are frequently involved in signal transduction in eukaryotic cells, these predicted structural features in VasX raise the possibility that it may imitate host cell proteins by utilizing its PH domain to attach itself to host cell structures such as biological membranes.

**VasX production depends on the** $\alpha^{54}$-dependent transcriptional activator VasH. Our finding that VasX was present exclusively in supernatants of VasH-producing *V. cholerae* (Fig. 1A) suggests that VasH regulates the production of VasX. This hypothesis is supported by our observations that VasX cannot be detected in V52ΔvasH culture supernatants by Western blotting with VasX antibody and that VasX levels are significantly reduced in pellet fractions (Fig. 2A). Importantly, DnaK, a cytoplasmic heat shock protein (22), was present only in bacterial pellet samples, indicating that the bacteria were intact, and thus, VasX is actively secreted and not released by cell lysis.

To investigate the relationship between VasH and the physically linked genes hcp and vasX (as depicted in Fig. 1C), we used Western blotting with polyclonal antibodies to detect Hcp and VasX in the complemented vasH deletion mutant. No Hcp and significantly reduced levels of VasX could be detected in pellets or concentrated supernatants when vasH was not expressed; however, upon induction of vasH expression, Hcp and VasX production within the cell and export/secretion into culture supernatants was restored (Fig. 2A). These data indicate that VasH plays an integral role in regulating both Hcp and VasX, supporting our hypothesis that hcp-2, vgrG-2, VCA0019, and vasX belong to the same T6SS regulon.

To further validate our data presented in Fig. 2A, we used semiquantitative RT-PCR to measure the vasX transcript levels in the complemented V52ΔvasH strain relative to the transcription of the outer membrane protein OmpW. It was previously demonstrated that the presence of arabinose does not affect the expression of OmpW (30). When vasH expression was induced with arabinose, vasX transcript levels increased ~10-fold (Fig. 2B). We propose that VasH acts directly at the promoter located immediately upstream of the T6SS satellite cluster encoding Hcp-2, VgrG-2, and VasX in *V. cholerae* V52. Our hypothesis is supported by work from Bernard et al., who recently demonstrated that VasH from *V. cholerae* O395 binds to promoters found upstream of the core T6SS gene cluster as well as promoters upstream of the satellite gene clusters (6).

**VasX secretion is dependent on the T6SS proteins Hcp, VasK, and VgrG-2.** Since VasX depends on the T6SS regulator VasH for expression, we hypothesized that the T6SS structural complex is required for VasX secretion. To test this, we employed the isogenic T6SS deletion mutants V52Δhcp-1.2 (V52 with hcp-1 and hcp-2 deleted), V52ΔvgrG-1, V52ΔvgrG-2, V52ΔvgrG-3, and V52ΔvasK (34). As shown in Fig. 3A, VasX was secreted by wild-type V52, V52ΔvgrG-1, and V52ΔvgrG-3 but not by V52ΔvasK, V52Δhcp-1.2, or V52ΔvgrG-2. Furthermore, the secretion of VasX matched the export pattern of Hcp. VgrG-2 was the only one of the three VgrG proteins absolutely required for the export and secretion of both Hcp and VasX (Fig. 3A). The absence of VasX in culture supernatants was not due to a failure to produce VasX within the cell, as VasX was present in bacterial pellet samples of all T6SS mutants tested (with the exception of the in-frame deletion mutant V52ΔvasX) (Fig. 3A).

Interestingly, deletion of vasX did not affect the production/export of Hcp (Fig. 3A), and thus, VasX does not appear necessary for the formation of the T6SS structural apparatus. Aside from Hcp nanotubes, VgrG protein trimers are also important factors for producing a functional T6SS cell-puncturing complex (33, 34). Therefore, we tested whether VasX was required for VgrG-1-mediated cross-linking of host cell actin. To determine whether host cell actin is cross-linked by V52ΔvasX, murine RAW 264.7 macrophages were infected with the wild type, V52Δhcp-1.2,
chromosomal copies of hcp V52 were deleted, and at least three experimental data sets. The localization of VasX and Hcp within the bacterium, we indicated antibody types (listed to the left of the blot). Molecular mass markers are shown to the right of both blots. Results are representative of at least three independent experiments.

**FIG. 3. Secretion of VasX is T6SS dependent.** (A) Western blot of VasX and Hcp in culture supernatants and bacterial pellets. Bacterial cultures were grown to late logarithmic phase; supernatant and pellet fractions were isolated and subjected to Western blotting using the indicated antibody types (listed to the left of the blot). Molecular mass markers are shown to the right of both blots. Results are representative of at least three independent experiments. (B) VasX is not required for T6SS-mediated covalent attachment of murine macrophage actin. RAW 264.7 macrophages were infected at a multiplicity of infection of 10 for 2 h. Cell lyses were subjected to SDS-PAGE followed by Western blotting using an antiaclin antibody. Molecular mass markers are shown to the left of the blot. The hcp deletion strain V52Δhcp-1,2 has both chromosomal copies of hcp deleted. Results are representative of at least three independent data sets.

V52ΔvasK, V52ΔvgrG-1, or V52ΔvasX for 2 h. Following infection, cells were collected, lysed, and resolved by SDS-PAGE followed by Western blotting using an antiactin antibody. Actin cross-linking was visualized by a "laddering" effect of actin bands, as monomeric units of G-actin are covalently attached to one another, forming higher-molecular-weight structures (33). As seen in Fig. 3B, only wild-type V52 and V52ΔvasX strains were capable of covalent cross-linking RAW 264.7 cell actin. Thus, deletion of vasX did not affect the assembly or the enzymatic function of the T6SS physical translocon complex. Taken together, we conclude from these data that VasK, Hcp, and VgrG-2 are required for VasX secretion and that VasX has the same transport dependencies as Hcp. Furthermore, VasX is not imperative for the formation of a functional T6SS translocon complex.

**VasX localizes to the bacterial membrane.** To investigate the localization of VasX and Hcp within the bacterium, we performed subcellular fractionation experiments. V52 was transformed with the β-lactamase-encoding plasmid pBAD24 for subcellular fractionation experiments to use β-lactamase as a periplasmic control. Other fractionation controls included DnaK (cytoplasm) and OmpU (membrane). As shown in Fig. 4, Hcp localized to all cellular compartments, whereas VasX localized specifically to the cytoplasmic and membrane fractions. It should be noted that Hcp was present in the periplasm at high abundance, while VasX could not be detected in this compartment.

**FIG. 4. VasX localizes to the bacterial membrane.** V52pBAD24 was grown to mid-logarithmic phase and subjected to subcellular fractionation. Various fractions (whole cell [WC], permeabilized V52 [PERM], membrane [M], periplasm [PP], and cytosol [CY]) were separated by SDS-PAGE followed by Western blotting with anti-VasX, -Hcp, -OmpU (membrane control), -DnaK (cytosol control), and β-lactamase (Bla; periplasm control) primary antibodies. Results are representative of three independent experiments.

**VasX is required for virulence toward the host model Dictyostelium discoideum.** We examined whether VasX is essential for virulence toward eukaryotes by using the D. discoideum host model system (9, 32, 39). In this plaque assay, V. cholerae was mixed with D. discoideum and plated on SM/5 nutrient agar (40), which is able to support the growth of bacteria but not the amoebae. To survive, amoebae must prey on the bacteria, resulting in plaque formation in the bacterial lawn. However, V. cholerae strains expressing a functional T6SS, such as V52, are virulent toward amoebae and can resist predation, thus preventing plaque formation (34). Importantly, T6SS-mediated virulence toward D. discoideum results in a loss of viable amoebae, as demonstrated by Pukatzki et al. (34). We performed a plaque assay with V52ΔvasX to determine whether this strain lost its virulence toward D. discoideum. As shown in Fig. 5A, no plaques were observed on plates with wild-type V52; however, plaques developed after a 4-day incubation with V52ΔvasX, V52ΔvasK, and V52ΔvgrG-1. As shown in Fig. 5B, the number of plaques in a lawn of V52ΔvasX was significantly less than those in lawns of V52ΔvasK and V52ΔvgrG-1 (P value < 0.001). V52ΔvgrG-1 exhibited a stronger plaque phenotype than V52ΔvasX; however, both VasX and VgrG-1 are required for T6SS-mediated virulence toward D. discoideum. To determine if VgrG-1 and VasX act synergistically, we created a vgrG-1 vasX double-knockout strain (V52ΔvgrG-1ΔvasX) and tested its plaque assay phenotype. As shown in Fig. 5B, the number of plaques in a lawn of V52ΔvgrG-1ΔvasX was not significantly different from that in a lawn of V52ΔvgrG-1 but did differ significantly from the number in a lawn of V52ΔvasX. Therefore, although VasX is important for T6SS-mediated virulence toward D. discoideum, VasX and VgrG-1 do not appear to act synergistically.

To verify that the ΔvasX phenotype is caused solely by the absence of vasX, we cloned vasX into the plasmid pBAD24 (15) downstream of an arabinose-inducible promoter (pBAD24-vasX) and introduced this plasmid into V52ΔvasX (resulting in strain V52ΔvasX/pBAD24-vasX) for complementation experiments. Overexpression of vasX in trans results in VasX secretion that is comparable to wild-type levels (data not shown). To confirm that VasX was responsible for
VasX interacts with membrane lipids via its PH domain. To test whether VasX can bind membrane lipids, we performed far-Western blotting using nitrocellulose membranes spotted with a variety of membrane lipids (Fig. 6A). Because subcellular fractionation experiments indicated that VasX localizes to the bacterial membrane, we tested whether VasX possessed the ability to bind bacterial lipopolysaccharide (LPS). Purified E. coli LPS (Sigma-Aldrich, St. Louis, MO) was spotted onto a PIP Strip at the same concentration as each of the membrane lipids on the strip (100 picomoles). In a separate experiment, the presence of LPS on the membrane was confirmed using an anti-lipid A polyclonal antibody (Abcam, Cambridge, MA) (data not shown). As shown in Fig. 6C, full-length VasX retained the ability to bind PA and each PIP as demonstrated in Fig. 6B; however, VasX did not bind to bacterial LPS.

To determine whether these interactions also occurred in aqueous solution, multilamellar vesicles (MLV) of PA, LPA, and each of the phosphatidylinositol phosphates (PIP). We also tested whether the first 200 residues of VasX, which encode the putative PH domain, retained the ability to interact with cell membrane phospholipids. As shown in Fig. 6B (left panel), VasX interacts with membrane phospholipids that bear a phosphorylated head group and two acyl chains, namely, phosphatidic acid (PA) and each of the phosphatidylinositol phosphates (PIP). However, VasX does not bind phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), lysophosphatidic acid (LPA), lysophosphocholine (LPC), or sphingosine-1-phosphate (S1P). We also tested whether the first 200 residues of VasX, which encode the putative PH domain, retained the ability to bind membrane lipids. As shown in Fig. 6B (right panel), VasX(1–200) exhibited the same lipid-binding pattern as full-length VasX. The positive control indicated in Fig. 6B represents purified protein (either full-length or truncated VasX) spotted directly onto the membrane.

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FIG. 6. VasX binds phosphoinositides but not LPS. (A) Schematic representation of the various biological membrane lipids present on PIP Strips, including phosphatidic acid (PA), phosphatidyl inositol (PI), phosphatidylinositol phosphate (PIP), phosphatidylyserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), and sphingosine-1-phosphate (SIP). (B and C) Purified full-length VasX or a truncated version consisting of residues 1 to 200 (containing the PH domain) was used to probe PIP Strips for lipid binding. Bound protein was detected using anti-6×His primary antibody and anti-mouse-HRP secondary antibody. The positive control (+) included purified VasX spotted directly onto the PIP Strip membrane. In panel C, E. coli LPS was spotted in place of the positive control on the PIP Strip. Results are representative of three independent experiments. (D) MLV pulldown of purified recombinant VasX and the N-terminal fragment of VasX(1–200). Full-length VasX, VasX(1–200), and BSA (negative control) were mixed with MLV of total liver extract (TLE), phosphatidic acid (PA), lysophosphatidic acid (LPA), or PBS (as a technical control) and divided into total (T), pellet (P), and supernatant (S) fractions by centrifugation. The partitioning of protein into each fraction was visualized by SDS-PAGE and Coomassie blue staining. Results are representative of three independent experiments.

clusions in a σ54-regulated T6SS regulon. The fact that VasH (encoded in the large T6SS gene cluster) was essential for the production of Hcp and VasX, both of which are encoded by a small auxiliary cluster, suggests that expression of genes within the large cluster supersedes that of the auxiliary gene clusters.

VasX secretion requires a functional T6SS, as V. cholerae mutants lacking the T6SS genes vasK, vgrG-2, and hcp-1,2 failed to secrete VasX into culture supernatants (Fig. 3A). In contrast, vgrG-1 and vgrG-3 mutants retained the ability to secrete VasX (Fig. 3A). The dependence of VasX secretion on VasK and Hcp was consistent with recent reports that VasK and Hcp are important for T6SS machine assembly and nanotube formation, respectively (4, 24). The observation that only VgrG-2 and not VgrG-1 or VgrG-3 was required for VasX secretion suggests that VgrGs differ in aspects other than their C-terminal effector domains. Furthermore, V52ΔvgrG-1 secreted less VasX than V52ΔvgrG-3. This suggests a VgrG protein hierarchy for the secretion of VasX into culture supernatants. Interestingly, the secretion of VasX from different T6SS mutants corresponds with the export of Hcp from the same strains. This implies a secretory relationship between VasX and Hcp and indicates that the Hcp nanotube is required for VasX secretion from the bacterium. Importantly, a polar effect of the vasK deletion was not responsible for the lack of secreted VasX, as VasX was present in V52ΔvgrG-2 pellets (Fig. 3A). We hypothesize that VgrG proteins possessing C-terminal extensions, such as the actin cross-linking domain of VgrG-1, are required exclusively for in vivo infections in order to provide an anchoring mechanism for T6SS effector delivery into the host cell. Therefore, VasX in vitro secretion requires only the core protein VgrG-2, which is also required to export Hcp into culture supernatants. Alternatively, the absence of VasX in V52ΔvgrG-2 supernatants might have been due to the lack of Hcp export from the cell. Both V52ΔvgrG-1 and V52ΔvgrG-3 retain the ability to export Hcp (33) and are therefore able to secrete VasX. As a consequence, VasX may strictly rely on Hcp for secretion from the bacterium.

Results of our plaque assays with D. discoideum indicated that VasX is required for virulence toward amoebae. The number of plaques that formed in V52ΔvasX lawns was significantly less than the numbers of plaques that developed in lawns of V52ΔvasK, V52ΔvgrG-1, and V52ΔvgrG-1ΔvasX. Even though VgrG-1 was dispensable for VasX secretion in vitro (Fig. 3A) and VasX is not required for the actin cross-linking activity of VgrG-1, both VgrG-1 and VasX are required for virulence toward amoebae (Fig. 5A and B) (23). Since V52ΔvgrG-1 and V52ΔvgrG-1ΔvasX have a more severe plaque-forming phenotype than V52ΔvasX, we conclude that V52ΔvgrG-1 has a dominant plaque phenotype and VasX and VgrG-1 do not appear to act synergistically in T6SS-mediated virulence.

Our bioinformatics analysis suggested that VasX possesses a PH domain (5, 19, 38). PH domains are defined by 100- to 200-amino-acid stretches that create a structural superfold typically implicated in binding phosphoinositides on cellular membranes (21). We demonstrated that VasX and a truncated version of VasX consisting of residues 1 to 200 (encompassing the putative PH domain) bind to membrane lipids, including various phosphatidylinositol phos-
phates and phosphatidic acid, by using two independent methods (Fig. 6). Because inositol phosphates are rarely found in bacteria (27), we postulate that the PH domain of VasX has a role in binding to host membrane lipids. Interference with host cell phosphoinositide metabolism and signaling by pathogenic bacteria is not uncommon and has been previously demonstrated for other enteric bacterial pathogens (8, 16, 31).

VasX is not required for the export of the T6SS apparatus component Hcp (Fig. 3A) or the enzymatic function of VgrG-1 (i.e., actin cross-linking) (Fig. 3B). In contrast, VasX requires Hcp and VgrG-2 for secretion into the extracellular milieu (Fig. 3A). Subcellular fractionation experiments identified VasX only in membrane and cytosolic fractions, with Hcp localizing to all bacterial compartments, including the periplasm. This allows us to speculate that VasX passes through the Hcp nanotube and bypasses the periplasm en route out of the bacterium, where it associates with the bacterial envelope. VasX detected in culture supernatants may be the result of being sloughed from the bacterial surface along with other T6SS structural proteins, such as Hcp and VgrG.

Far-Western blots indicated that VasX binds phosphoinositides but not bacterial LPS. Although we cannot rule out that VasX binds to other structurally related lipids in the bacterial envelope, we speculate that VasX associates with the T6SS apparatus to contact host membranes and function as an effector protein. VasX may be translocated into the bacterial envelope, we speculate that VasX associates with other T6SS structural proteins, such as Hcp and VgrG.

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