RESEARCH ARTICLE

Secretome Analysis of *Vibrio cholerae* Type VI Secretion System Reveals a New Effector-Immunity Pair

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ABSTRACT **The type VI secretion system (T6SS) is a dynamic macromolecular organelle that many Gram-negative bacteria use to inhibit or kill other prokaryotic or eukaryotic cells. The toxic effectors of T6SS are delivered to the prey cells in a contact-dependent manner. In *Vibrio cholerae*, the etiologic agent of cholera, T6SS is active during intestinal infection. Here, we describe the use of comparative proteomics coupled with bioinformatics to identify a new T6SS effector-immunity pair. This analysis was able to identify all previously identified secreted substrates of T6SS except PAAR (proline, alanine, alanine, arginine) motif-containing proteins. Additionally, this approach led to the identification of a new secreted protein encoded by VCA0285 (TseH) that carries a predicted hydrolase domain. We confirmed that TseH is toxic when expressed in the periplasm of *Escherichia coli* and *V. cholerae* cells. The toxicity observed in *V. cholerae* was suppressed by coexpression of the protein encoded by VCA0286 (TsiH), indicating that this protein is the cognate immunity protein of TseH. Furthermore, exogenous addition of purified recombinant TseH to permeabilized *E. coli* cells caused cell lysis. Bioinformatics analysis of the TseH protein sequence suggest that it is a member of a new family of cell wall-degrading enzymes that include proteins belonging to the YD repeat and Rhs superfamilies and that orthologs of TseH are likely expressed by species belonging to phyla as diverse as *Bacteroidetes* and *Proteobacteria*.**

IMPORTANCE **The Gram-negative bacterium *Vibrio cholerae* causes cholera, a severe and often lethal diarrheal disease. The 2010-2012 epidemic in Haiti and new explosive epidemics in Africa show that cholera remains a significant global public health problem. The type VI secretion system (T6SS) is a dynamic organelle expressed by many Gram-negative bacteria, which use it to inject toxic effector proteins into eukaryotic and bacterial prey cells. In this study, we applied a comparative proteomics approach to the *V. cholerae* T6SS secretome to identify new substrates of this secretion apparatus. We show that the product of the gene VCA0285 is likely a new peptidoglycan hydrolase that is secreted by T6SS and that its cognate immunity protein is encoded by the gene that is immediately downstream (VCA0286). Bioinformatics analysis shows that VCA0285 carries four conserved motifs that likely define a large family of hydrolases with antibacterial activity. The identification of new antibacterial T6SS effectors provides useful information for the development of novel antibiotics and therapeutic agents.**

Received 20 January 2015 **Accepted** 27 January 2015 **Published** 10 March 2015

Citation Altindis E, Dong T, Catalano C, Mekalanos J. 2015. Secretome analysis of *Vibrio cholerae* type VI secretion system reveals a new effector-immunity pair. mBio 6(2): e00075-15. doi:10.1128/mBio.00075-15.

Editor Rino Rappuoli, Novartis Vaccines and Diagnostics, Siena, Italy

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This article is a direct contribution from a Fellow of the American Academy of Microbiology.

Protein targeting and secretion mechanisms are some of the most specialized and interesting cellular processes of microbes. Gram-negative bacteria have evolved six secretion systems to translocate proteins from the cytoplasm to the extracellular milieu or into neighboring cells. The type VI secretion system (T6SS) is the most recent example of these secretion systems and plays an important role in competition between bacterial species in complex communities, as well as in evading host defenses during infection (1). It was functionally identified in *Vibrio cholerae* through the genetic definition of its main components and canonical substrates, but it is encoded by more than 25% of Gram-negative species that have been so far been sequenced at the genome level (2). Bioinformatics and structural studies have shown that T6SS proteins are functionally analogous to T4 bacteriophage tail proteins, including the tail spike, tube, sheath, and base plate (3). Utilizing this intracellular organelle, bacteria can translocate effector proteins into both prokaryotic and eukaryotic cells (4). Basler et al. showed that VipA and VipB proteins form a dynamic tubular sheath that switches between extended and contracted states within the bacterial cytosol (5). This contraction powers the secretion of hemolysin-coregulated protein (Hcp), valine-glycine repeat protein G (VgrG), and other effectors into neighboring cells to kill them or inhibit their growth (6). The Hcp protein forms an inner tube composed of stacked hexameric rings within the VipA-VipB sheath, while VgrG proteins and PAAR (proline, alanine, alanine, arginine) motif-containing proteins form a spikelike structure that decorates the end of the Hcp tube (1, 7, 8).

In recent years, a number of T6SS effector proteins have been identified by using various proteomics, bioinformatics, and genomics approaches (1). These effectors can be classified according to their targets. There are different VgrG effectors which target eukaryotic cells. Of those, VgrG-1 of *V. cholerae* inhibits actin polymerization and, thus, the phagocytosis process (9). Similarly, the C-terminal domain of *Aeromonas hydrophila* VgrG-1 causes cell rounding and apoptosis in HeLa cells (10). Likewise, PldA and PldB, phospholipases of *Pseudomonas aeruginosa*, activate the Akt-phosphatidylinositol (PI) pathways and promote the invasion of nonphagocytic cells (11). On the other hand, effectors that target bacterial cells can be classified into three groups: cell wall-degrading enzymes, membrane-targeting proteins, and nucleases (12). The best-characterized cell wall-targeting effectors are the Tse1 and Tse3 proteins of *P. aeruginosa*, which function in peptidoglycan hydrolysis (13). Additionally, the VgrG-3 carboxyl domain in *V. cholerae* was also able to degrade peptidoglycan and likely acts as a muramidase (14, 15). VasX of *V. cholerae* and BTH_12691 of *Burkholderia thailandensis* are members of a large family of effectors that target the membrane (16). Recent studies revealed a third group of effectors that degrade nucleic acids in prey cells. For example, the secretion of nucleases RhsA and RhsB by *Dickeya dadantii* is dependent on T6SS and the VgrG-3 protein, and the expression of these proteins in *Escherichia coli* cell cytoplasm causes DNA degradation and growth inhibition (17). The T6SS apparatus of *V. cholerae* is quite versatile in that it can be used to kill eukaryotic cells, such as *Dictyostelium discoideum* amoebae or macrophage cell lines (18), as well as prokaryotic cells, such as *E. coli*, by deploying effectors with different enzymatic activities (14, 15).

The characterization of PAAR proteins prompted the suggestion of the multiple effector translocation VgrG (MERV) model for how toxic effectors are loaded onto the T6SS spike and then translocated into cells (7). A modified version of this model (1) encouraged us to use a mass spectrometry-based approach coupled with bioinformatics to investigate new effectors secreted by T6SS. Proteomics approaches have been successfully applied to identify new enzymatic effectors of T6SS for different bacteria, including *P. aeruginosa*, *Burkholderia thailandensis*, *Serratia marcescens*, *A. hydrophila*, and *Flavobacterium johnsoniae* (4, 13, 16, 19, 20). Recently, we employed a gel-free, in-solution-digestion proteomics method to characterize the outer membrane vesicles (OMVs) of *V. cholerae* (21). Here, we used this powerful approach to compare the active and inactive T6SS (T6SS$^+$ and T6SS$^-$, respectively) secretomes to identify a new secreted effector of *V. cholerae* T6SS.

RESULTS

Identification of T6SS-secreted proteins. To better analyze the differences between the secretomes of active and inactive states of T6SS and to identify novel secreted substrates of the *V. cholerae* T6SS, we applied a mass spectrometry-based method of secretome analysis (21). This approach allows high-throughput comparison of complex protein mixtures in a gel-free manner. The secreted proteins produced by the ΔFlgG *V. cholerae* strain 2740-80 (which has an active T6SS but is deficient in production of flagella) were compared to those produced by different isogenic T6SS mutant strains. Two different T6SS mutant strains, *V. cholerae* 2740-80 ΔFlgG ΔClpV and ΔFlgG ΔClpV Δgp25, were used in this experimental design. In order to avoid having cytoplasmic contamination, we collected secretome samples from cells that were grown to late exponential phase (optical density at 600 nm [OD_{600}] of 1.0). The proteins present in such samples from two different biological replicates of each strain/mutant were then analyzed by liquid chromatography-tandem mass spectrometry (LC–MS-MS).

By setting a cutoff of 2 or more mapped peptides per individual protein identified, 103 individual proteins were designated secreted proteins (Fig. 1A and B; see Table S1 in the supplemental material). Samples from each replicate showed good technical and biological reproducibility. Of the 103 secreted proteins identified, 18 were predicted to be extracellular. Compared with the theoretical proteome of *V. cholerae*, only 44 of 2,536 cytoplasmic proteins were identified, most of which were abundant ribosomal proteins. Because OMVs were removed by ultracentrifugation before the analysis, the protein content of the secretome was consistent with the theoretical mechanism of secretion and did not contain outer membrane or periplasmic proteins. Only 3 of 73 outer membrane proteins and 10 of 97 periplasmic proteins of *V. cholerae* were identified in the secretome. PSORTb (22) could not make a cellular localization prediction for 33 proteins that were identified as potentially secreted proteins (Fig. S1).

The total number of spectral count values, (i.e., the number of total peptides identified for each protein) was used to determine whether each protein is differentially secreted by T6SS (23). We identified 9 secreted proteins present only in the secretome of the wild type and not in those of the two T6SS mutants (Fig. 1A and B; Table 1). These include all previously reported T6SS substrates except for the PAAR proteins. The PAAR repeat proteins of *V. cholerae* are quite small (9 kDa and 18 kDa) and may not be detected because of their small size and low expression levels under these *in vitro* growth conditions. As expected, Hcp was the most abundant protein in the two samples prepared from the T6SS$^+$ strain (168 and 173 total peptides, respectively) but this protein was virtually absent in the samples prepared from the two T6SS mutants. The list of T6SS substrates also included all three VgrG proteins and the T6SS effectors VasX and TseL. The detection of all these previously identified T6SS substrates in the T6SS$^+$ secretome validates our experimental approach.

A new zinc binding protein, encoded by VCA0065, is expressed when T6SS is inactive. A protein encoded by VCA0065 was the only protein (≥3 total peptides) identified specifically in the two T6SS$^-$ mutant secretomes (Table 1). This 84-kDa protein is predicted to be a member of the peptidase gluzincin family and carries conserved zinc binding motifs. Recently, Hood et al. used proteomics to explore the T6SS of *P. aeruginosa* and identified three proteins present only in the inactive state of T6SS (13). Of those three proteins, the two encoded by PA3422 and PA1888 do not have any homologs in *V. cholerae*. However, the third (encoded by PA3836) is predicted to be a zinc binding protein as well. Interestingly, PAAR proteins also have conserved zinc binding histidine residues (7).

Identification of a new T6SS effector protein, encoded by VCA0285. The only protein identified in the T6SS secretome that was not previously known to be a T6SS substrate was encoded by the gene VCA0285. This hypothetical protein was identified via two different mapped peptides (Fig. 2A). Interestingly, VCA0285 is located downstream from a gene encoding a PAAR protein

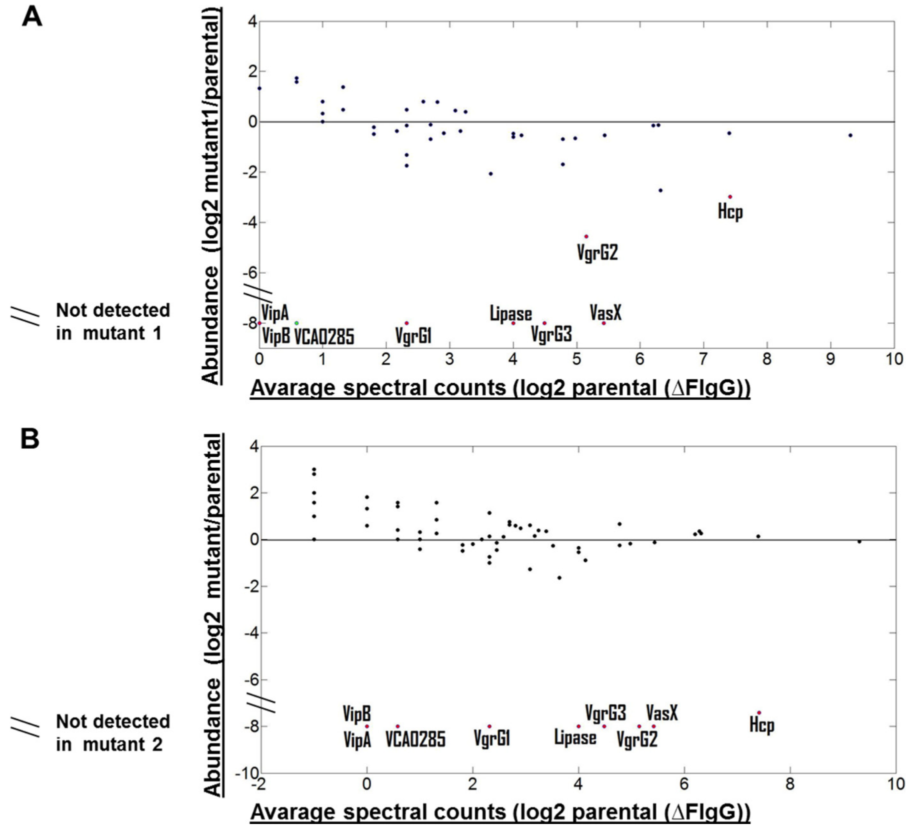

FIG 1 Comparative secretome analysis identifies a novel *V. cholerae* T6SS effector candidate. Comparison of individual proteins in the parental strain (ΔFlgG) to those in mutant 1 (ΔFlgG ΔClpV) (A) and mutant 2 (ΔFlgG ΔClpV Δgp25) (B) using quantitative proteomics. A MatLab application was used to compare the average spectral counts (total peptide number) of each protein to analyze the abundance in different samples. The abundances of proteins around the range from −2 to 2 are approximately the same. Previously identified T6SS substrates that were not identified in the mutant secretomes are indicated by red dots. TseH, the new effector identified in this study, is shown by a green dot. Proteins below −6 were not identified in the mutants' secretomes but only in the parental strain's secretomes.

(VCA0284). The predicted 25-kDa protein encoded by VCA0285 is relatively small in comparison with other T6SS substrates, and the HHpred tool predicts that it carries a putative amidase or peptidase domain (Fig. 2B). Homology searches of the hypothetical protein encoded by VCA0285 revealed 130 bacterial proteins that showed significant similarity. These orthologs could be classified into two groups that were encoded by species belonging to either the *Bacteroidetes* (16 proteins) or *Proteobacteria* (113 proteins, of which 98 were from different *V. cholerae* strains) (see Table S2 in the supplemental material). The product of VCA0285

TABLE 1 List of T6SS-related *V. cholerae* proteins identified by comparative proteomics analysis of secretomes derived from *V. cholerae* 2740-80 mutants

		No. of peptides identified in secretome of indicated strain[b]					
		Wild type (ΔFlgG)		ΔFlgG ΔClpV1 mutant		ΔFlgG ΔClpV1 Δgp25 mutant	
Protein[a]	Locus	R1	R2	R1	R2	R1	R2
Hcp	A1F1I3_VIBCL	168	173	15	28	1	1
VasX	A1F1I0_VIBCL	43	43	0	0	0	0
VgrG-2 protein	A1F1I2_VIBCL	34	37	1	2	0	0
VgrG-3 protein	A1F481_VIBCL	18	27	0	1	0	0
Lipase	A1F9V9_VIBCL	15	17	0	0	0	0
VgrG-1 protein	A1F9V6_VIBCL	5	5	3	1	0	0
VipA	A1F466_VIBCL	0	2	0	0	0	0
VipB	A1F467_VIBCL	0	2	0	1	0	0
Hypothetical protein (VCA0285)	A1FA05_VIBCL	2	1	0	0	0	0
Hypothetical protein (VCA0065)	A1F7Y2_VIBCL	0	0	0	3	5	4

[a] Protein names were extracted from UniProt. The first 9 proteins are putative T6SS-dependent proteins, while VCA0065 was only identified in the T6SS mutants.
[b] Each experiment was performed with two different biological repeats (R1 and R2). 0, no peptides were detected for the protein.

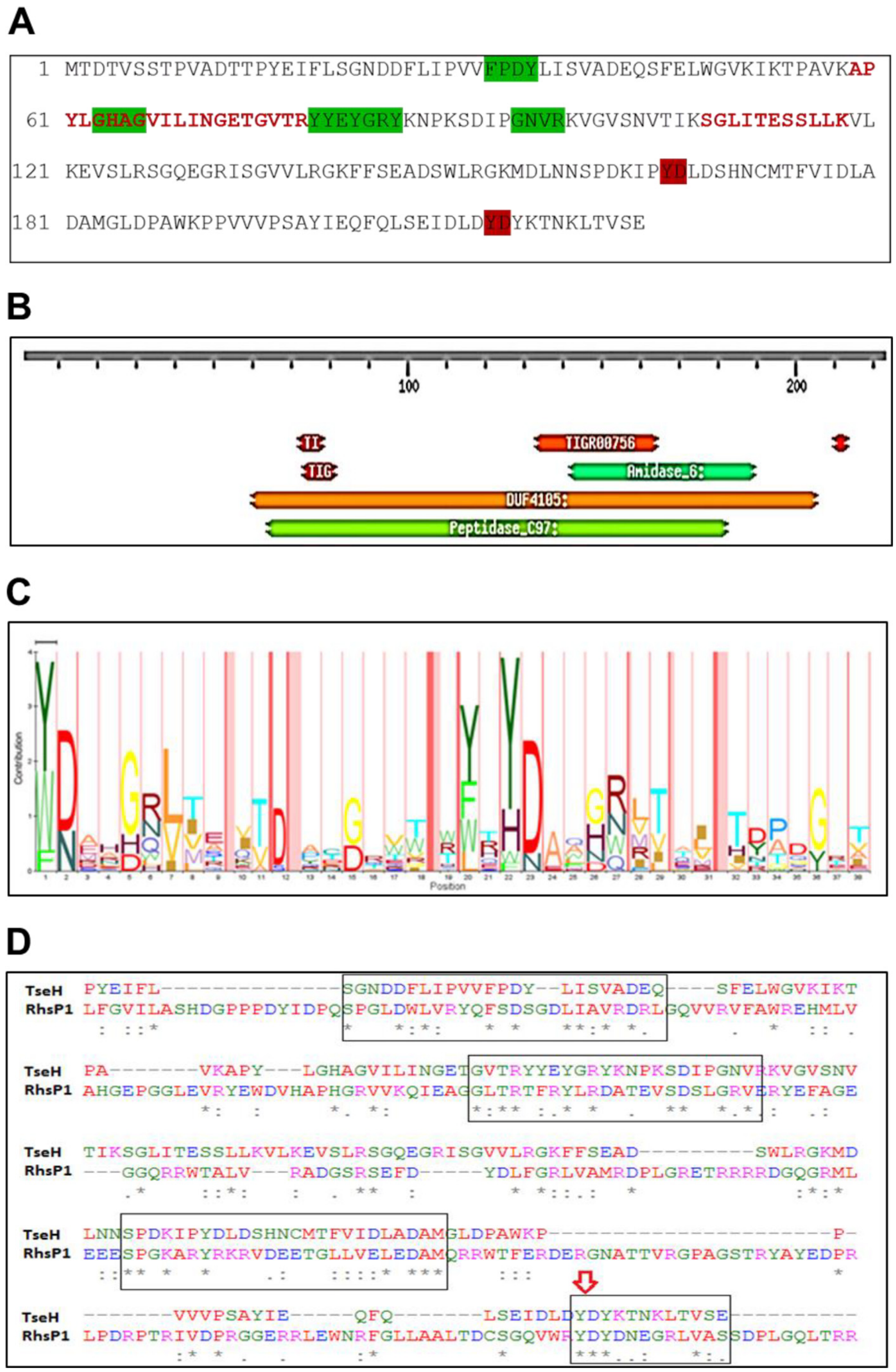

FIG 2 Amino acid sequence of the novel T6SS effector TseH and bioinformatics analysis. (A) The peptides of TseH identified by mass spectrometry are in red font. The green highlighting defines the four conserved motifs in 62 BLASTp homologs of TseH. Red highlighting shows the YD repeat regions of the protein. GHAG region is the conserved catalytic motif among different cell wall amidases. (B) HHpred analysis reveals an amidase (amidase_6) or peptidase (peptidase_C97) domain for the TseH protein. (C) Conserved motif of YD repeats generated from the Pfam database. (D) Clustal Omega alignment of TseH and RhsP1. Boxes indicate the most conserved regions between two proteins. The arrow points to the conserved YDY residues. The asterisks indicate identical residues, double dots indicate strong similarity and the single dots indicate weaker similarity. Red indicates small, blue indicates acidic, magenta indicates basic, green indicates Hydroxyl + sulfhydryl + amine and grey indicates unusual amino acids.

shares ~30% identity with most of the hypothetical Blast homologs, and a large number of these include proteins annotated as "rearrangement hotspot (Rhs)" or "YD repeat-containing proteins." Genes encoding Rhs and YD repeat proteins are frequently found linked to genes encoding VgrG and Hcp proteins (24). Rhs genes have recently been shown to encode functional toxins that are inhibitory in a contact-dependent manner (17). VCA0285 carries two YD residue repeats (Fig. 2A). While the space between the

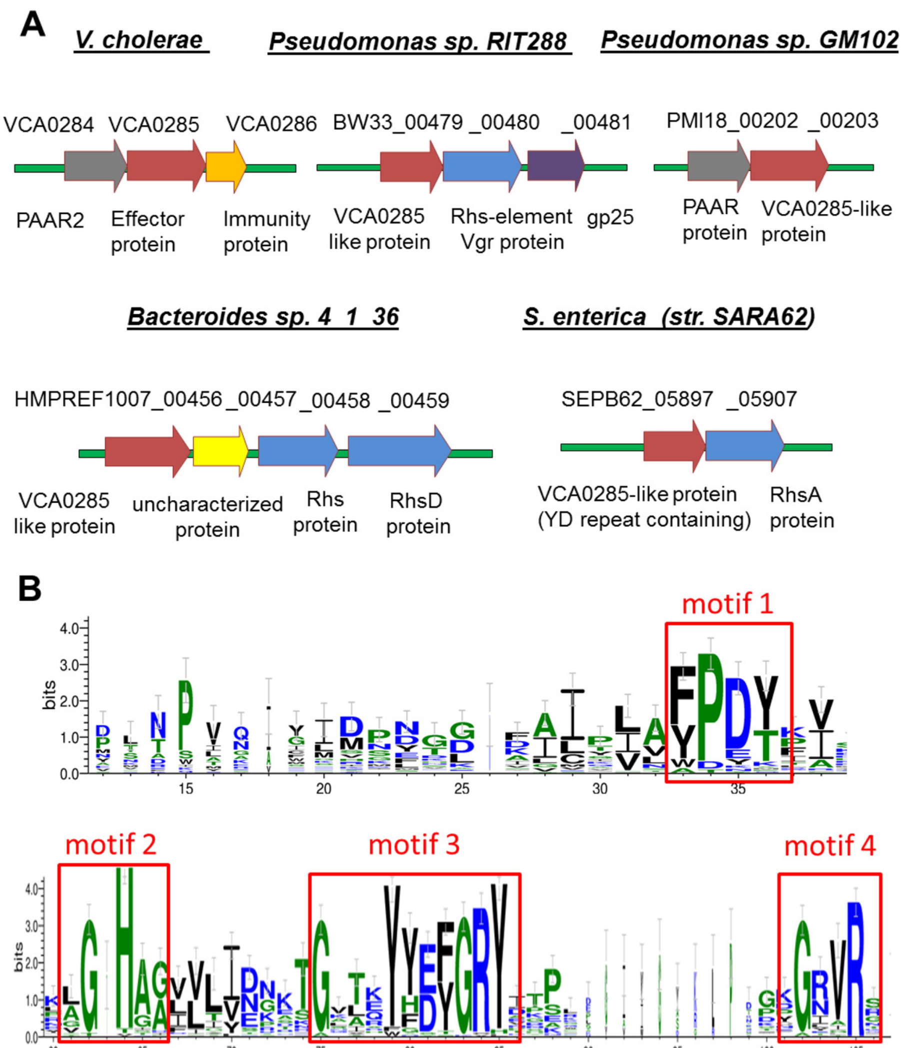

FIG 3 Genomic arrangements and conserved motifs of TseH orthologs. (A) Genomic arrangement of TseH ortholog proteins from different species. Colors indicate candidate effector (red), neighboring PAAR proteins (grey), candidate immunity protein (orange), Rhs proteins (blue), and gp25 (purple). (B) WebLogo sequence alignment of TseH orthologs identified with BLASTp. The four conserved motifs are shown by boxes. The accession numbers of protein sequences used to prepare this WebLogo diagram are listed in Table S2 in the supplemental material.

two YD residues in the classical, canonical YD repeat motif is about 21 to 22 amino acid residues (Fig. 2C), the space between the YD repeats in the VCA0285 gene product is 45 residues. Very recently, Hachani et al. showed that the VgrG1c gene of *P. aeruginosa* is involved in the delivery of the Rhs-related protein toxin RhsP1, which carries YD repeats (25). Intriguingly, we detected a conserved region around YD residues of the VCA0285-encoded product and the RhsP1 protein (Fig. 2D). To provide further evidence that these orthologs might be T6SS effectors, we checked their locations in their respective genomes for synteny with other T6SS-related genes. Indeed, strains including *Pseudomonas* sp. strain GM102, *Pseudomonas* sp. strain RIT288, *Bacteroides* sp. strain 4_1_36, and *Salmonella enterica* carry genes encoding orthologs of the VCA0285 product downstream from genes encoding predicted PAAR or Rhs proteins (Fig. 3A). Rhs proteins include many that have been implicated as T6SS effectors because they frequently display PAAR domains or require VgrG proteins for their secretion (7, 17). To further explore this relatedness, we used the Clustal Omega and WebLogo3 sequence alignment tools to search for common motifs among homologs of the protein encoded by VCA0285 that could be identified by employing the Blast search algorithm (26–28). This search revealed four different conserved motifs for 61 different Blast homologs of VCA0285 (Fig. 3B). Of great interest, motif 2 is conserved within different cell wall amidases and is one of the shared catalytic motifs of amidases, i.e., GHAA, GHTG, and GHVA (16). Although the predicted product of VCA0285 does not carry the conserved Rhs domain PXXXXDPXGL (29), when we limited our Blast sample group to five Rhs proteins of *S. enterica* that are homologous to the product of VCA0285, this analysis revealed that all four main motifs were conserved between the predicted VCA0285 product and these Rhs proteins (Fig. S2). Collectively, these results suggested that the product of VCA0285 and these Rhs proteins with motif 2 and other amidase motifs are likely all secreted T6SS effectors that are probably hydrolases. Therefore, we named this novel effector type six effector hydrolase (TseH).

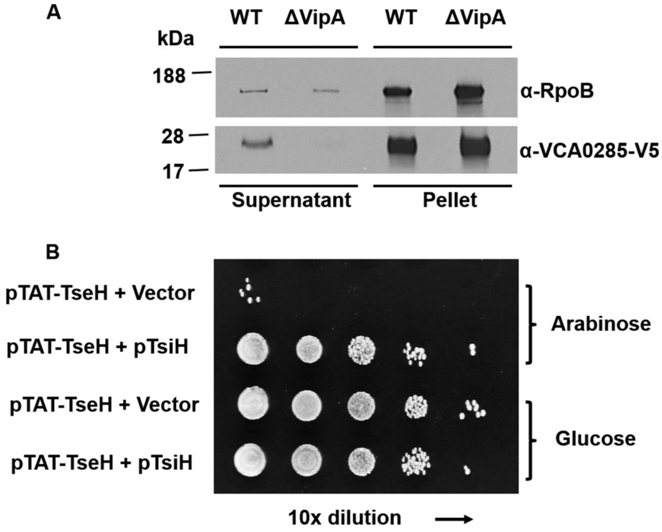

FIG 4 TseH is secreted in a T6SS-dependent manner, and TsiH is the cognate immunity protein. (A) Western blot analysis of the supernatants and the whole-cell pellets of *V. cholerae* V52 wild type (WT) and the ΔVipA strain carrying the pBAD18 vector encoding VCA0285 with a 3×V5 epitope tag. Protein secretion was tested by using tag-specific antibody. The RNA polymerase subunit RpoB was used to control the level of cell lysis. (B) TseH is toxic in the periplasm of *E. coli*, and the toxicity is neutralized by coexpression of the cognate immunity protein TsiH. Gene expression was induced by arabinose and repressed by glucose in the medium.

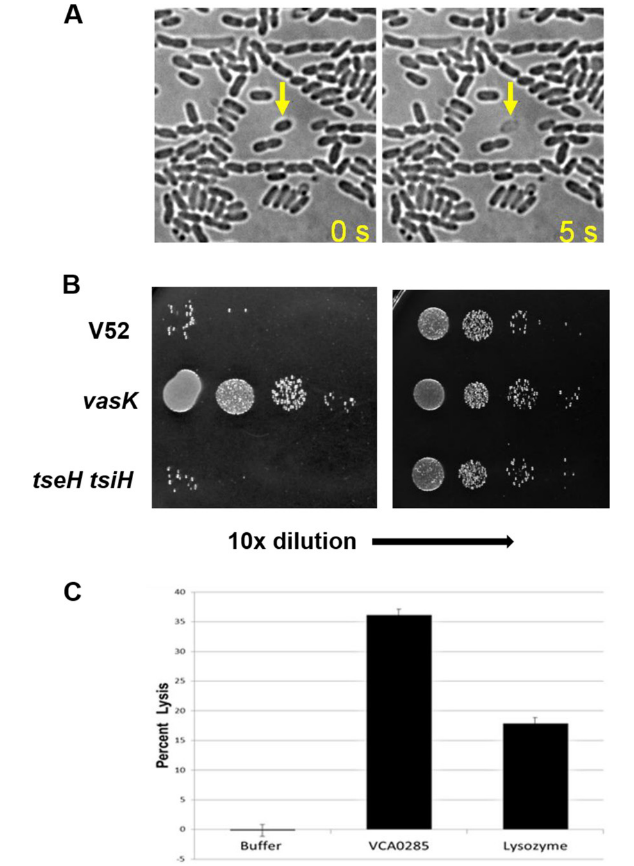

FIG 5 TseH is toxic to *V. cholerae*. (A) Light microscopic imaging of *V. cholerae* expressing TseH in the periplasm of the Δ*tseH* Δ*tsiH* double mutant. Yellow arrows highlight cells with a spherical shape that are seen frequently when TseH is expressed in the periplasm. Left, cells before induction; right, cells after 5 s of induction. (B) Effect of TseH on T6SS-mediated killing. Left, *E. coli* survival when *E. coli* cells were mixed with the wild type, the Δ*vasK* mutant, and the Δ*tseH* Δ*tsiH* mutant of *V. cholerae* V52; right, the prey is the Δ*tseH* Δ*tsiH* mutant. (C) Addition of exogenous TseH to *E. coli* leads to cell lysis. Results are the means of three independent experiments. Error bars represent the standard deviations derived from all data points.

To confirm that the secretion of TseH depends on T6SS, we cloned TseH into an L-arabinose-inducible vector, pBAD18, with a 3×V5 epitope tag and tested its secretion in wild-type *Vibrio cholerae* strain V52 or the V52 ΔVipA mutant. Consistent with our mass spectrometry results, Western blot analyses of cell and supernatant fractions showed that TseH was detectable in the supernatant of the wild-type strain but not in that of the ΔVipA strain (Fig. 4A). These results show that the secretion of TseH is dependent on T6SS and can be blocked by deactivating T6SS.

The expression of TseH in the periplasm is toxic to *E. coli*, and the VCA0286 gene product suppresses its toxicity. Based on our secretome and bioinformatics analyses, we hypothesized that TseH is a new T6SS effector of *V. cholerae*. Because TseH carries a predicted amidase domain, we reasoned that the expression of TseH might be toxic in *E. coli* if it hydrolyzes peptide cross-links in the peptidoglycan. The expression of TseH in the cytosol had little effect on *E. coli*'s survival (data not shown). However, when TseH was expressed in the periplasm using a twin-arginine delivery signal sequence (30), *E. coli* was readily killed (Fig. 4B). T6SS antibacterial effectors often exist in pairs with antagonistic immunity proteins (31). Thus, we tested whether the product of the downstream gene VCA0286 can neutralize the toxicity of TseH. Indeed, the expression of VCA0286 protected *E. coli* from the toxic effect of periplasmically expressed TseH, indicating that VCA0286 likely encodes the immunity protein to TseH (Fig. 4B). Accordingly, we named this immunity protein type six immunity hydrolase (TsiH).

We next tested whether the expression of TseH is toxic in *V. cholerae* in the absence of TsiH. We constructed a double knockout mutant lacking TseH and TsiH and expressed TseH in the periplasm of the resulting mutant. Microscopic analysis showed multiple cell bursting events in cells of the *V. cholerae* mutant lacking the VCA0286-encoded TsiH (Fig. 5A; see Movies S1 to S3 in the supplemental material). We conclude that TseH is indeed a toxic T6SS effector that likely targets the bacterial cell wall for degradation and that TsiH is its cognate immunity protein.

TseH is not essential for T6SS function or optimal prey cell-killing activity. Given the critical location of the TseH and TsiH genes, just downstream from the PAAR protein-encoding VCA0284, we speculated that the inactivation of TseH might affect the T6SS functions, including prey cell killing and protein secretion. In *V. cholerae* strain V52, deletion of the gene encoding TseH did not lead to any change in T6SS-dependent killing of *E. coli*, indicating that TseH is not essential for T6SS's killing function (Fig. 5B, left). Additionally, wild-type V52 could not outcompete the double mutant lacking TseH and TsiH, suggesting that the delivery of TseH might not be efficient under the conditions used or that TseH might have preferred target organisms other than nonimmune strains of *V. cholerae* (Fig. 5B, right).

Previous studies have shown that T6SS effectors can target the peptidoglycan in prey cells for degradation (13, 14). For example,

TABLE 2 Identification of three new ΔTseH mutant-specific proteins by comparative proteomics analysis of *V. cholerae* V52 strains

		No. of peptides identified in secretome of indicated strain[b]					
		Wild type			ΔVCA0285 mutant		
Locus	Annotation[a]	R1	R2	R3	R1	R2	R3
A1EJ54_VIBCL	Zinc binding ABC transporter	0	0	0	8	8	5
A1EPS5_VIBCL	Amino acid binding ABC transporter	0	0	0	1	4	1
A1EPV5_VIBCL	CysP, thiosulfate binding ABC transporter	0	0	0	3	1	1

[a] Protein annotations are from UniProt.
[b] Each experiment was performed with three different biological repeats (R1 to R3). 0, no peptides were detected.

Brooks et al. recently reported that exogenous addition of the VgrG-3 C-terminal subunit to *E. coli* cells leads to lysis (15). Accordingly, we tested TseH in an analogous assay. Recombinant TseH was purified and then added to *E. coli* cells that had been treated with polymyxin B in order to permeabilize their outer membrane and give TseH direct access to peptidoglycan within the periplasmic space. Hen egg white lysozyme and buffer alone were used as positive and negative controls, respectively. While the buffer had no lysis activity, we observed that TseH induced approximately 35% of the permeabilized cells to lyse, compared to ~20% lysis for lysozyme-treated cells (Fig. 5C). These data strongly support the conclusion that TseH is an enzyme that catalyzes degradation of the target cell's peptidoglycan.

Disruption of TseH results in secretion of 3 new proteins. To further evaluate the function of TseH in secretion, we next explored whether TseH was important for the production of some proteins detected in the secretome. In this experiment, we did not exclude the OMVs. In total, 119 proteins were identified from the TseH mutant and its wild-type parental V52 strain in three biological replicates for each strain (see Table S3 in the supplemental material). When we compared these proteins with the ΔFlgG mutant's secretome in which OMVs were excluded, we realized that the differentially identified proteins were OMV specific. The disruption of TseH did not affect the secretion of T6SS substrates, since all previously reported substrates except PAAR proteins were detected. On the other hand, using this comparative screen, we identified 3 new proteins (≥3 total peptides each) present in the secretomes of mutant cells that were absent in that of the wild type (Table 2). Classification of these three proteins, VCV52_1056 (ABC transporter), VCV52_0504 (CysP, thiosulfate ABC transporter), and VCV52_1340 (amino acid ABC transporter) according to their predicted functional categories revealed three new ABC transporters. Whether the occurrence of these proteins in the ΔTseH secretome reflects changes in gene expression or an alteration in the cell envelope architecture in the absence of TseH is a topic that would require further investigation.

Interestingly, Hood et al. identified three proteins that were present only in the inactive state of *P. aeruginosa*'s T6SS (13). Of those three proteins, PA3836 is the homolog of VCV52_1056 (VC1101, 50% identity), whose product was identified only in the secreted proteins produced by the ΔTseH mutant. The VCV52_1056 and PA3826 proteins are both zinc binding ABC transporters. Thus, in *P. aeruginosa*, loss of T6SS function also results in the increased expression of proteins involved in zinc homeostasis.

DISCUSSION

Various approaches have been applied to identify new effectors of T6SS through bioinformatics, but the number of functionally identified effectors is still somewhat limited (1, 7, 16). Recently, Shneider et al. identified PAAR motif-containing proteins as possible secreted T6SS effectors (7). According to the MERV model, these authors proposed that VgrG/PAAR spikes can be decorated by cargo proteins that are themselves T6SS effectors. This and the modified MERV model, where effectors are also loaded into the Hcp tube (1, 19, 32), encouraged us to attempt to identify secreted effectors that did not display PAAR or VgrG-homologous sequences. Using a proteomics approach coupled with bioinformatics and genetic analysis, we discovered the TseH and TsiH effector-immunity pair for *V. cholerae*'s T6SS. This effector-immunity pair is conserved within all sequenced *V. cholerae* strains in the UniProt database. Furthermore, we were able to identify all previously identified substrates of T6SS by this approach (Table 1). The only proteins that could not be detected were the PAAR proteins, which may have escaped detection due to their low expression levels or small molecular mass. The results presented here show the striking ability of TseH to kill *E. coli* when expressed in the periplasm or when exogenously added to *E. coli* cells that have been permeabilized. The toxic effect of TseH can be neutralized by coexpression of the TsiH immunity protein in the periplasm. Microscopic analysis shows that the expression of TseH in the periplasm of a V52 mutant lacking TsiH causes cell bursting, suggesting that TseH can indeed damage the bacterial cell wall in the absence of TsiH (Fig. 5A; see Movies S1 to S3 in the supplemental material).

The properties of TseH overlap those of the main T6SS effectors. TseH does not carry a predicted signal sequence and is a small protein composed of 223 amino acids (~24 kDa) which can potentially fit the 40-Å internal pore of the Hcp hexamer tube. Our bioinformatics analyses of TseH and TsiH revealed other interesting aspects related to the gene organization of this effector-immunity pair. The genes encoding TseH-TsiH are located just downstream from VCA0284, which encodes the larger of the two PAAR proteins identified so far in *V. cholerae* (7). This PAAR protein carries a transthyretin domain (TTR) that may be used as an adaptor to decorate the VgrG tip with other effectors (7). Whether TseH interacts with the VCA0284-encoded PAAR-2 protein cannot be definitively established from our data and would require additional biochemical and/or genetic studies. Interestingly, Dong et al. recently reported that the VCA0284-to-VCA0286 genes are regulated by RpoN, a sigma factor that is activated by VasH, a coregulatory protein that is encoded by the main T6SS gene cluster (31). Together, these observations suggest that TseH, TsiH, and the PAAR-2 protein would all likely be coordinately expressed with the genes encoding the products needed for assembly and function of the T6SS organelle.

The domain organization of the PAAR proteins consists of different extension domains, including YD and Rhs repeats and other enzymatic domains (1). BLAST analysis shows that genes encoding other TseH homologs are frequently located downstream from genes encoding PAAR proteins or Rhs proteins

(Fig. 3A). It is also important to note that most of the annotated BLASTp homologs of TseH were in fact Rhs- or YD repeat-containing proteins. Indeed, WebLogo sequence alignment of TseH BLASTp homologs revealed four conserved motifs which are also conserved in Rhs proteins of *S. enterica* (see Fig. S2 in the supplemental material). Although most Rhs proteins are predicted to be comparatively large proteins carrying internal Rhs repeats, there are hundreds of proteins annotated as Rhs proteins in the UniProt database that are smaller than 250 amino acids. Similar to the results for TseH, bioinformatic analyses of Rhs proteins indicate that they commonly carry potentially toxic sequences on their C-terminal ends, such as putative hydrolase, amidase, peptidase, and nuclease domains (24). Recently, Koskiniemi et al. reported the VgrG-dependent secretion of two Rhs proteins (RhsA and RhsB) of *Dickeya dadantii* strain 3937 (17). Because these Rhs proteins also carry PAAR motifs, it has been proposed that these proteins are likely secreted by associating with VgrG spike complexes (7). The PAAR domain-containing Tse5 and Tse6 effectors of *P. aeruginosa* require VgrG proteins for stability and secretion (19), which is again consistent with the MERV model for effector secretion (7). YD repeats of Rhs proteins have been previously proposed to be associated with carbohydrate binding, and this in turn may be important to the recognition of peptidoglycan chains for effectors that attack the bacterial cell wall (24). Thus, the presence of YD repeats in orthologs of TseH is consistent with its postulated activity as a peptidoglycan hydrolase. A recently identified YD repeat-containing toxic T6SS effector of *P. aeruginosa*, RhsP1, is delivered by VgrG1c (25). The conserved motif around the YD residues of TseH and RhsP1 (Fig. 2D) might be a common feature that allows these effectors to engage very diverse substrates that have as a common feature polysaccharide or glycan components (e.g., peptidoglycan, nucleic acids, or glycolipids). It is formally possible that YD repeats are also involved in binding T6SS effectors to heterotrimers of VgrG complexed with PAAR proteins (7). Other T6SS effectors bind to the center hole of Hcp hexamers, suggesting that their secretion may require their loading into the lumen of the Hcp tube in the assembled, extended form of the T6SS organelle (19, 32). According to our modified MERV model (1, 7) and in accordance with other data reported by Mougous and colleagues (19, 32), TseH could also be carried within the assembled Hcp tube as well. Unfortunately, assembling such complexes *in vitro* to test this hypothesis is not technically feasible at the time.

Our bioinformatics analysis also revealed that TseH belongs to a family of putative T6SS effectors encoded by conserved genes present in members of the *Bacteroidetes* and *Proteobacteria* (see Table S2 in the supplemental material). This is interesting given the considerable evolutionary distance between these two phylums (Table S2). Recently, Russell et al. provided evidence for the *in vivo* active secretion of T6SS effectors by *Bacteroides fragilis* (4). Thus, if TseH orthologs are secreted by *Bacteroidetes* species, these observations would suggest that an ancestral gene for this effector may have been transferred from a pathogen (e.g., *V. cholerae*) to a commensal (e.g., *B. fragilis*) or vice versa during gut cocolonization.

Lastly, our results regarding the expression of a zinc-dependent protease when T6SS is inactive and the expression of three new ABC transporters when TseH is disrupted could be helpful to understand the regulation of *V. cholerae* T6SS. Interestingly, both VC274080_A0096 (VCA0065) and VCV52_1056 (VC1101) are predicted to be zinc binding proteins. It is well documented that T6SS gene expression is regulated by divalent metals, including zinc and iron, in other species (33, 34). The depletion of iron, zinc, and other nutrients is sensed by bacteria as a signal to upregulate the expression of virulence genes, including the T6SS genes (35). The homolog of VCV52_1056 (VC1101), PA3836, was reported to be one of the three proteins secreted only when T6SS is inactive in *P. aeruginosa* (13). This implies that although the absence of TseH does not affect the T6SS secretion or the killing of *E. coli*, it has an indirect effect similar to that of the absence of *P. aeruginosa* T6SS. Why bacteria upregulate zinc binding proteins or other new ABC transporters in the absence of T6SS is a new question to be answered by the T6SS research community. The zinc binding ability of PAAR proteins may be an interesting clue to help answer this question (7).

In conclusion, the data presented here provide strong evidence that TseH is a member of a large family of T6SS effectors encoded by genes that are widely distributed among different bacterial species. Because the hydrolytic activity of TseH likely targets peptidoglycan, our results suggest that members of this conserved effector family play important roles in antagonistic bacterium-bacterium interactions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used are listed in Table S4 in the supplemental material. Luria Bertani (LB) medium at 37°C was used for normal growth conditions. The following antibiotics and chemicals were added to the medium when appropriate: kanamycin (50 μg/ml), ampicillin (100 μg/ml), streptomycin (100 μg/ml), and arabinose (0.1%). Mutants and gene expression vectors were constructed as described previously (14). All constructs were verified by sequencing.

Secretome preparation and mass spectrometry. *V. cholerae* strains were grown to an optical density at 600 nm (OD_{600}) of 0.8. The cells were removed, and supernatant was filtered through 0.22-μm-pore-size filters. Protease inhibitors were added to filtrates to inhibit protein degradation. OMVs were removed by ultracentrifugation (130,000 $\times$ *g* for 4 h at 4°C) for T6SS comparative analysis. Culture supernatants were mixed with 10% tricarboxylic acid overnight and then washed with ice-cold acetone for optimal precipitation. The precipitated proteins were resuspended in 0.5 M urea. In-solution digestion and protein sequence analysis by mass spectrometry were performed as described previously (21). OMVs were not removed from the secretomes of the wild type and the ΔTseH mutant.

Bioinformatics. Peptide sequences (protein identity) were determined by matching the protein databases of *V. cholerae* strains V52 and 2740-80 with the acquired fragmentation patterns using Sequest software (Thermo Fisher, San Jose, CA). The amino acid sequence of *V. cholerae* TseH (UniProt ID Q9KMN9_VIBCH) was analyzed using (i) HHpred to identify conserved domains, (ii) BLASTp to identify homologs, (iii) Clustal Omega to check the alignments between the homologs, and (iv) WebLogo 3 to identify conserved motifs using the alignments. MatLab was used to compare the abundance of proteins between different samples and to produce the first figure based on a previously described method (16). The sequence logo for the conserved motif of YD repeats was generated using the hidden Markov model profile in the Pfam database (36).

T6SS-dependent-killing assay. Killing assays and Western blot experiments were performed as described previously (14). Briefly, a 10:1 ratio was used to mix cultures of predator and prey strains, respectively, and the cultures were spotted onto LB medium for 3 h. Bacterial spots were cut and washed into 1 ml of LB. The prey strains' survival was measured by serial dilution on selective medium. anti-RpoB antibody was used to check cell lysis, while anti-V5 epitope antibody was used to assay the secretion of TseH.

Microscopic analysis. Samples were prepared as previously described (14). Cells carrying pBAD-VCA0285 were grown to exponential phase (OD_{600} of 0.6) and induced with arabinose for 3 h at 37°C. Cells were concentrated 10 times by centrifugation and spotted onto a 1% agarose pad. A Nikon Ti-E microscope was used for taking time-lapse microscopic images.

Exogenous addition of TseH. The recombinant TseH was purified as previously described by Davies et al. (37). *E. coli* strain SM-10 was used for the lysis assay. *E. coli* cells were grown to an OD_{600} of 0.8. Polymyxin B was added to *E. coli* cells for outer membrane permeabilization. The effect of recombinant TseH was determined by measuring the absorbance of the cells (OD_{600}) at 0 and 10 min (15).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00075-15/-/DCSupplemental.

Table S1, PDF file, 0.5 MB.
Table S2, PDF file, 0.2 MB.
Table S3, PDF file, 0.4 MB.
Table S4, PDF file, 0.1 MB.
Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.3 MB.
Movie S1, AVI file, 0.5 MB.
Movie S2, AVI file, 5.3 MB.
Movie S3, AVI file, 1.5 MB.

ACKNOWLEDGMENTS

We thank Marek Basler and Brian Ho for providing T6SS mutants of *V. cholerae*. Thanks to the members of J.J.M.'s laboratory for helpful discussions. Thanks to Ross Tomaino (Taplin Mass Spectrometry Facility) for his technical contributions and Erman Korkut for MatLab software analysis.

This work was supported by National Institute of Allergy and Infectious Diseases grant AI-01845 (to J.M.).

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