VasH Is a Transcriptional Regulator of the Type VI Secretion System Functional in Endemic and Pandemic *Vibrio cholerae*[†]

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The Gram-negative bacterium *Vibrio cholerae* is the etiological agent of cholera, a disease characterized by the release of high volumes of watery diarrhea. Many medically important proteobacteria, including *V. cholerae*, carry one or multiple copies of the gene cluster that encodes the bacterial type VI secretion system (T6SS) to confer virulence or interspecies competitiveness. Structural similarity and sequence homology between components of the T6SS and the cell-puncturing device of T4 bacteriophage suggest that the T6SS functions as a molecular syringe to inject effector molecules into prokaryotic and eukaryotic target cells. Although our understanding of how the structural T6SS apparatus assembles is developing, little is known about how this system is regulated. Here, we report on the contribution of the activator of the alternative sigma factor 54, VasH, as a global regulator of the *V. cholerae* T6SS. Using bioinformatics and mutational analyses, we identified domains of the VasH polypeptide that are essential for its ability to initiate transcription of T6SS genes and established a universal role for VasH in endemic and pandemic *V. cholerae* strains.

Vibrio cholerae, the etiological agent of the diarrheal disease cholera, remains a major health risk in developing countries, with approximately 120,000 deaths annually (49). The organism is classified into more than 200 serogroups based on its lipopolysaccharide O antigens, but only strains of the O1 serogroup have been associated with pandemics. The seventh, current pandemic O1 El Tor *V. cholerae* biotype (5) replaced the O1 classical biotype that was responsible for the sixth pandemic and most likely for the first five pandemics (4, 40). Since its appearance in southeastern India and the Bay of Bengal in 1992 (3), strains of the O139 serotype have spread to a large portion of the Asian subcontinent and are considered to have pandemic potential (17). O1 and O139 serogroup strains utilize two major virulence factors: toxin-coregulated pilus (TCP) and cholera toxin (CT). In contrast, many non-O1 and non-O139 strains of *V. cholerae* are capable of eliciting diarrheal or nonintestinal diseases in a CT/TCP-independent manner (33, 43). *V. cholerae* often employs accessory toxins such as hemolysin (HlyA) and actin cross-linking repeats in toxin (RtxA) (10). We believe that the type VI secretion system (T6SS) is one of the accessory factors used by *V. cholerae* to export effector molecules across its cell wall to confer cytotoxic effects on host cells.

Three gene clusters—one large cluster (VCA0107 to VCA0124) and two auxiliary clusters (VCA0017 to VCA0022 and VC1415 to VC1416)—collectively encode the *V. cholerae* T6SS. Two copies of *hcp* and three copies of *vgrG* (*vgrG1-3*)

gene are commonly found in T6SS gene clusters (29, 36, 42, 46, 50) and are believed to form the translocon conduit (35). Hcp is predicted to form the inner tube of the envelope translocon conduit which is decorated with a trimeric VgrG cap (20).

Virulence of *V. cholerae* toward eukaryotic phagocytes, including *Dictyostelium discoideum*, and murine J774 and RAW264.7 macrophages requires a functional T6SS (23, 35, 36). *Pseudomonas aeruginosa* uses its T6SS to inject bacteriolytic enzymes into the periplasm of neighboring bacteria to degrade the peptidoglycan layer unless the targeted bacterium produces specific inhibitors for these enzyme effectors (39). We recently reported that the *V. cholerae* T6SS mediates contact-dependent killing of *Escherichia coli* and other Gram-negative bacteria (24). Thus, *V. cholerae* may use the T6SS to compete with other marine bacteria or grazing amoebae in the environment or with enteric bacteria and invading immune cells in the human intestine.

The role of the T6SS in the human disease cholera is not understood, and elucidation of its role is complicated by the fact that this system is not expressed in pandemic El Tor strains under laboratory conditions (27). Non-O1/non-O139 *V. cholerae* strains expressing the T6SS constitutively under laboratory conditions have been isolated and are presently being examined (51; also D. Provenzano, University of Texas, Brownsville, TX, personal communication). We employ the endemic *V. cholerae* O37 serogroup strain V52 as a model to dissect T6SS expression because this pathogenic strain expresses its T6SS constitutively (36). Though pandemic *V. cholerae* O1 El Tor strains C6706 and N16961 carry the full T6SS gene complement (27), the regulatory gene products of *luxO* and *tsrA* prevent constitutive T6SS expression (51). Therefore, pandemic *V. cholerae* strains may have evolved mechanisms to suppress constitutive T6SS expression, but the environmental cues required for T6SS activation in these strains remain to be discovered.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference or source
Strains		
<i>V. cholerae</i> V52	O37 serogroup strain; $\Delta hpaA \Delta trxA \Delta hlyA$ Sm ^r	34
<i>V. cholerae</i> N16961	El Tor O1; Sm ^r	Mekalanos laboratory, Harvard Medical School
<i>V. cholerae</i> O395	Classical O1, Ogawa; Sm ^r	Mekalanos laboratory, Harvard Medical School
<i>E. coli</i> DH5 α λ pir	λ I ⁻ ϕ 80d <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁻) <i>supE44 thi-1 gyrA relA1</i>	Provenzano laboratory, University of Texas at Brownsville, TX
<i>E. coli</i> SM10 λ pir	<i>thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu pir</i> Km ^r	Mekalanos laboratory, Harvard Medical School
<i>E. coli</i> GM2163	F ⁻ <i>dam-13::Tn9</i> (Cam ^r) <i>dcm-6 hsdR2</i> (r _K ⁻ m _K ⁺) <i>leuB6 hisG4 thi-1 araC14 lacY1 galK2 galT22 xylA5 mlr-1 rpsL136</i> (Str ^r) <i>fhuA31 tsx-78 glnV44 mcrA mcrB1</i>	Fermentas
<i>E. coli</i> MG1655	F ⁻ λ ⁻ <i>ilvG rfb-50 rph-1</i>	Raivio laboratory, University of Alberta
<i>E. coli</i> XL10 Gold	<i>endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac Hte</i> Δ (<i>mcrA</i>)183 Δ (<i>mcrCB-hsdSMR-mrr</i>)173 Tet ^r [F ⁺ <i>proAB lacI^aZ</i> Δ M15 Tn10(Tet ^r) Amy Cm ^r]	Stratagene
<i>E. coli</i> BL21*(DE3)	F ⁻ <i>ompT hsdSB</i> (r _B ⁻ m _B ⁻) <i>gal dcm rne-131</i> (DE3)	Taylor laboratory, University of Alberta
Plasmids		
pBAD24	pBAD vector, pBR322 ori, <i>araC</i> , Amp ^r	14
pWM91	<i>oriR6K mobRP4 lacI ptaC tnp mini-Tn10</i> Km; Km ^r Amp ^r	25
pGEM-T Easy	Vector for cloning PCR products; Amp ^r	Promega

In a previous report, we suggested that *vasH* encodes an activator of the alternative sigma-54 (σ^{54}) factor allowing VasH and σ^{54} (encoded by *rpoN*) to act in concert to control transcription of the *V. cholerae* T6SS Hcp gene (36). Ishikawa et al. recently found that the expression of Hcp was growth phase dependent in the *V. cholerae* O1 strain A1552 and involved the quorum-sensing regulators HapR and LuxO, RpoN, and the cyclic AMP receptor protein (CRP) global transcriptional regulatory complex (16). We recently demonstrated that VasH, in addition to controlling the expression of *hcp-1* and *hcp-2*, regulates the downstream gene *vasX* (VCA0020), which encodes a lipid-binding virulence factor (26). Bernard et al. recently demonstrated that recombinant VasH binds the promoters that drive expression of the large T6SS cluster (VCA0107 to VCA0124) and the satellite cluster starting with VCA0017 (*hcp-1*) (2). *In vivo* reconstitution experiments in *E. coli* demonstrated that VasH was required to initiate transcription of these promoters (2). Here, we report the characterization of an attenuated mutant in *V. cholerae* strain V52 with an in-frame deletion in *vasH* (VCA0117) (36). The Δ *vasH* mutant was unable to produce Hcp, the “hallmark event” of T6SSs, and failed to engage in T6SS-mediated virulence toward *D. discoideum* (36). To better understand how the T6SS contributes to the pathogenic behavior of endemic and pandemic *V. cholerae* strains, we subjected *vasH* to mutational analysis and identified domains essential for the ability of VasH to activate transcription at T6SS promoters.

MATERIALS AND METHODS

Strains and culture conditions. Strains and plasmids used in this study are summarized in Table 1. Unless stated otherwise, all bacteria were grown shaking (200 rpm) in Luria-Bertani (LB) broth at 37°C. Antibiotic concentrations to maintain the plasmids were 100 μ g $\cdot$ ml⁻¹ ampicillin or 50 μ g $\cdot$ ml⁻¹ kanamycin. *E. coli* strains DH5 α λ pir and SM10 λ pir were used for cloning and mating purposes, respectively. *D. discoideum* AX3 cells, obtained from the Dicty Stock Center (Chicago, IL), were maintained in liquid culture (HL5) with shaking (150 rpm) at 22°C.

DNA sequence analysis and protein structure prediction analysis. All nucleotide sequence analyses and alignments were performed with MacVector, version 11.0.2. VasH protein structure prediction was performed using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), HHpred (<http://toolkit.tuebingen.mpg.de/hhpred>), and Pfam (<http://pfam.sanger.ac.uk/>). Phylogenetic analysis was performed using the online tool Phylogeny.fr (www.phylogeny.fr) (7). One hundred bootstrap data sets were analyzed to evaluate the significance of the tree.

In-frame deletions and plasmid construction. An in-frame deletion of *vasH* (VCA0117) was generated using the method described by Metcalf et al. (25). Genomic DNA from *V. cholerae* strain V52 was used as the template to amplify regions flanking the *vasH* gene (*vasH*_{V52}) with the primer pairs VCA0117A/VCA0117B and VCA0117C/VCA0117D (oligonucleotide sequences are listed in Table 2). PCR products resulting from primer combinations A/B and C/D were stitched together by overlapping PCR. The resulting knockout construct for *vasH* was digested with BamHI and cloned into the suicide plasmid pWM91 (25). For controlled expression of epitope-tagged VasH, *vasH* was PCR amplified from *V. cholerae* V52 chromosomal DNA using primers 5'-VasH and 3'-myc-VasH with unique restriction sites. The resulting PCR product was subcloned into pGEM-T Easy vector (Promega) and transformed into *E. coli* strain GM2163. pGEM-T Easy-VasH_{V52}::myc was digested with EcoRI and XbaI and the *vasH*::myc fragment was subcloned into the expression vector pBAD24 (14). *vasH*_{V52} with a deletion of the putative N-terminal regulator domain (*vasH*_{V52- Δ N}) and *vasH*_{V52} with a deletion of the C-terminal helix-turn-helix (HTH) motif (*vasH*_{V52- Δ HTH}) were generated in the same manner using the primer pair 3'-myc-VasH and VasH _{Δ N} and the pair 5'-myc-VasH and VasH _{Δ HTH}, respectively.

Site-directed mutagenesis of VasH. Amino acid substitutions were introduced into VasH using a QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. Primers used to introduce point mutations are summarized in Table 2. The PCR-amplified plasmid containing the mutation was then transformed into XL10 Gold *E. coli* cells. Each mutation was confirmed by DNA sequencing.

Protein secretion profiles. Overnight cultures of each bacterial strain were diluted to 1:100 in 3 ml of fresh LB containing appropriate antibiotics and grown until they reached the mid-logarithmic phase of growth (optical density at 600 nm [OD₆₀₀] of ~0.6) with or without 0.1% (wt/vol) L-arabinose to induce expression from the P_{BAD} promoter in pBAD24. Bacterial cultures were centrifuged at 14,000 $\times$ g for 5 min to separate cells from supernatants. Pelleted bacteria were resuspended in equal volumes of gel loading buffer (40% glycerol, 0.24 M Tris-HCl, pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% β -mercaptoethanol) and boiled for 10 min. Culture supernatant volumes of 300 μ l were filtered through 0.22- μ m-pore-size low-protein-binding polyvinylidene fluoride (PVDF) syringe filters (Millipore), precipitated with 20% trichloroacetic acid (TCA) for 15 min on ice, pelleted by centrifugation at 14,000 $\times$ g for 5 min at

TABLE 2. Oligonucleotide sequences

Primer name and function	Direction ^a	Sequence ^b
DNA manipulation		
VCA0117-A		GGATCCAGCAGTATCAAGCAC
VCA0117-B		CAATTTCAAGCACTTGTAGGCCAGCCATTGACTCATGGTTAC
VCA0117-C		GTAACCATGAGTCAATGGCTGGCCTACAAGTGCCTGAAATTG
VCA0117-D		GAATTCATCTGGCTGAAAAAT
5' VasH		GAATTCACCATGAGTCAATGGCTGGCG
3' myc VasH		CCTCTAGATCATAAATCTTCTTCAGAAATTAATTTTTGTTCTG
		GGGTTTTGATCTCCAA
5' myc VasH		GAATTCACCATGGAACAAAAATTAATTTCCGAAGAAGATTTA
		AGTCAATGGCTGGCGTTT
VasH _{ΔN}		CCGAATTCATGTTATCGCGTACTTT
VasH _{ΔHTH}		CCTCTAGATCAACTGACCGCCTGTTT
Point mutation		
Walker A	F	GCCACTGGCACGGGTGCAGAGTTAGTCGC
	R	GCGACTAACTCTGCACCCGTGCCAGTTTC
Walker B	F	GCAGCGTATTTTTGGCTGAGATCGGAG
	R	CGCGCATCTCAGCCAAAAATAGCGTGC
qRT-PCR		
<i>hcp-2</i> (VCA0017)	F	TGTGAAATGCCCACTGCCAAGAC
	R	GCGTTAACGTGGTCCCAAGTGATT
<i>vgrG-2</i> (VCA0018)	F	TGATATCGACTTTCTGCACCGCCT
	R	TAGAGGGTATGTTTGCCCGCTTCA
<i>vasW</i> (VCA0019)	F	TAACTGGGTGAGCTTGGTTCCAGA
	R	GGCTGATTTGAGCACCATCAACCA
<i>vasX</i> (VCA0020)	F	TCCTTTACCCAAAGCGGATCGACA
	R	GCCATCACGCAGTTGCCTTAAAGT
<i>vasH</i> (VCA0117)	F	ATGCATTTGACCGCGGAACACTTG
	R	AGCAAGATCGAGTGTACTTGGCGA
<i>vasK</i> (VCA0120)	F	AACTGGGTTCAATGCCAAACGACC
	R	AACCACGCTTGGTGAGCATTTGAG
<i>ompW</i> (VCA0867)	F	GGACTTGCTGCTAACGTTGGCTTT
	R	CCTGCTTTGTAGGTTGCCGTTGTT

^a F, forward; R, reverse.^b Restriction sites are underlined.

4°C, and washed twice with ice-cold acetone. Protein pellets from supernatants were resuspended in 40 µl of SDS-PAGE loading buffer and boiled for 10 min. Samples were subjected to SDS-PAGE (10% acrylamide) and analyzed by Western blotting using rabbit polyclonal antibody against VasX (diluted 1:1,000) (26), anti-DnaK (diluted 1:15,000; Stressgen), anti-Myc (Sigma, diluted 1:1,000), and anti-Hcp (diluted 1:500) (23). Blots were probed with goat anti-mouse horseradish peroxidase (HRP) and goat anti-rabbit HRP (both diluted 1:1,000) secondary antibodies before development.

Semiquantitative Western blot band intensity analysis. Overnight bacterial cultures were diluted 1:100 in LB medium containing the appropriate antibiotics. Arabinose was added to the cultures to final concentrations of 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, and 1%, and the cultures were incubated at 37°C with shaking until an OD₆₀₀ of 0.8 was obtained. Pellet fractions from overnight cultures were also retained. Pellet fractions were normalized based on optical densities. Samples were boiled for 10 min in SDS gel buffer and subjected to 10% SDS-PAGE followed by Western blot analysis using anti-Myc, anti-VasX, anti-DnaK, and anti-Hcp primary antibodies. Secondary antibodies included donkey anti-rabbit IRDye 800CW (Li-Cor) and donkey anti-mouse IRDye 680 (Li-Cor). Western blots were scanned using the Li-Cor Odyssey Infrared Imager, and band quantification was performed using the Odyssey Infrared Imaging System application software (version 3.0).

***D. discoideum* plaque assays.** One hundred microliters of bacterial (grown for 16 h at 37°C) and *D. discoideum* (1 × 10³ AX3 cells) cultures was plated on SM/5 plates (34). Arabinose (0.1%) was added to SM/5 plates when indicated. The numbers of plaques were counted after plates were incubated at 22°C for 3 days.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). Bacterial cultures were grown to mid-logarithmic phase of growth in the presence or absence of 0.1% L-arabinose at 37°C with shaking (200 rpm). Total RNA was

extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Extracted RNA was frozen in RNase-free water at -80°C until use.

RNA concentrations were determined using a NanoDrop spectrophotometer (Thermo Scientific), and 1 µg of RNA from each sample was subjected to DNase I (Invitrogen) treatment to remove residual DNA, followed by a reverse transcription reaction using random primers and SuperScript III Reverse Transcriptase (Invitrogen). Semiquantitative real-time PCR was performed (40 cycles of a two-step cycle followed by a melting curve) with PerfeCTa SYBR Green FastMix (Quanta) according to the manufacturer's instructions. Three sets of primers against VCA0017 (*hcp-2*), VCA0018 (*vgrG-2*), VCA0019 (hypothetical protein), VCA0020 (*vasX*), VCA0120 (*vasK*), VCA0117 (*vasH*), and VCA0867 (*ompW*) were designed using PrimerQuest from Integrated DNA Technologies (IDT). Primers were tested for performance in an RT-qPCR with a cDNA concentration gradient, and those with slopes of between -3.3 and -3.7, efficiency of ~1.0, and R² of ~1.0 were selected and used in the RT-qPCR studies (primer sequences used in the study are summarized in Table 2). *ompW* (VCA0867) encoding a bacterial outer membrane gene was used as a reference because the expression of this gene is unaffected by arabinose (30). The transcriptional fold change of each gene was determined by the comparative threshold cycle (C_T) number using Mastercycler EP Realplex system (Eppendorf) and then calculated using the 2^{-ΔΔC_T} method (41) in Microsoft Office Excel. Each experiment was performed in triplicate.

***E. coli* killing assay.** The protocol was adapted from MacIntyre et al. (24). Briefly, rifampin-resistant *E. coli* strain MG1655 and rifampin-sensitive *V. cholerae* were mixed at a multiplicity of infection (MOI) of 10 with volumes normalized by OD₆₀₀ readings. A total of 25 µl of the mixed bacterial culture was spotted onto predried LB agar and incubated at 37°C for 4 h. Bacteria were harvested, and the numbers of CFU per ml of surviving prey and predator were

FIG. 1. Polymorphic VasH carries three conserved domains. (A) Schematic diagram of VasH. The protein is composed of 530 amino acids and is predicted to have three functional domains depicted here: an N-terminal regulator domain, a central domain with two Walker motifs (alignments of Walker boxes of several σ^{54} -dependent activators are shown), and a C-terminal DNA binding domain (HTH). Numbers represent amino acid residues. (B) Phylogenetic tree of polymorphic *vasH* in *V. cholerae*. The nucleotide sequences of *vasH* from 26 sequenced strains of *V. cholerae* were used to generate a neighbor-joining tree. The number of bootstrap data sets was set to 100; bootstrap values are listed for each branch. Serogroups of each strain are listed; N/A, information is not available.

measured by serial dilution and selective growth on agar containing 50 $\mu\text{g} \cdot \text{ml}^{-1}$ rifampin and 100 $\mu\text{g} \cdot \text{ml}^{-1}$ streptomycin, respectively.

RESULTS

VasH is a sigma-54 activator protein with conserved domains. Bioinformatic analysis of VasH with the basic local alignment search tool (BLAST) revealed important signatures shared with bacterial enhancer binding proteins (bEBPs), including a σ^{54} activator domain in the center of VasH (E value, $2.2\text{E}-150$) that harbors conserved Walker A and Walker B motifs (Fig. 1A). Walker motifs are essential bEBP elements whose ATP binding and hydrolysis activities (15, 18) provide energy to mediate the change of the σ^{54} -RNA polymerase (RNAP) holoenzyme complex to the open conformation (12, 38). We further identified a C-terminal helix-turn-helix (HTH) DNA binding motif with an E value of $3.6\text{E}-07$ and a putative N-terminal regulatory domain (Fig. 1A). The N-terminal regulatory domains of bEBPs can act as intramolecular repressors or activators (8, 13). The HHpred prediction algorithm (44) suggested that VasH carries a GAF domain (small ligand binding domain) in the first 200 amino acids (E value of 0.0012) similar to the GAF domains of NorR (47) and NifA (1). Sequence alignments of 27 σ^{54} bEBPs revealed that the HTH motif in this protein family shares the general conserved sequence AL-X₉-AA-X₂-LG (where X represents any amino acid) with a few mismatches (28). VasH carries RL-X₉-AA-X₂-LG between residues 496 and 512 in its predicted HTH motif, manifesting only a single mismatch from the conserved sequence. Our bioinformatic analysis combined with the find-

ing by Bernard et al. that VasH binds T6SS promoters (2) suggests that VasH drives σ^{54} -mediated transcription of T6SS genes.

The role of VasH in pandemic strains. The genome of the pandemic El Tor strain N16961 encodes a T6SS gene complement similar to V52. Because we noticed differences in the nucleotide sequence of *vasH* between these two strains, we evaluated the polymorphic nature of *vasH* by assembling a phylogenetic tree with the nucleotide sequences of *vasH* from strains with sequenced genomes (Fig. 1B). We found that *vasH* was highly polymorphic; strains of O1 and O139 serogroups clustered together while non-O1/non-O139 were dispersed due to strain-specific single nucleotide polymorphisms (SNPs). While the *vasH* genes from O1 El Tor strain N16961 (*vasH*_{N16961}) and O139 strain MO10 are identical, *vasH* genes from the O37 strains V52 and MZO-3 differ from each other. The *vasH* genes of the 26 *V. cholerae* strains investigated had an identical length of 1,593 bp but differed in 44 nucleotides (2.8%). Out of the 44 nucleotide substitutions, 34 resulted in synonymous (silent) mutations while 10 substitutions led to nonsynonymous mutations. The nonsynonymous mutations were not equally distributed along the VasH polypeptide but, rather, clustered in the putative N-terminal regulatory domain and in the linker region between the σ^{54} activator domain and the HTH domain. Furthermore, the 123-nucleotide-long portion of *vasH* that encodes the HTH domain is identical in all *V. cholerae* strains included in this analysis, suggesting that substitutions are not tolerated. The central activator domain also appears to be highly conserved; all 20 nucleotide dif-

FIG. 2. Episomal expression of VasH_{V52} induces Hcp synthesis but not secretion in N16961. (A) Expression of VasH_{V52}::myc establishes Hcp and VasX synthesis as shown by Western blotting. Each bacterial strain was grown in LB medium containing ampicillin in the presence or absence of arabinose. Mid-logarithmic-phase cells were separated into pellet and supernatant fractions and subjected to SDS-PAGE followed by Western blotting with anti-VasX, anti-Hcp, anti-Myc, anti-Bla, and anti-DnaK antibodies. (B) Plaque assay showing that overexpression of VasH_{V52} does not restore virulence of N16961 Δ vasH. Approximately 1×10^3 mid-logarithmic *D. discoideum* cells were plated onto SM/5 nutrient agar plates (with or without 0.1% arabinose) with 100 μ l of bacterial strains. Pictures were taken on the third day of incubation at 22°C. (C) Expression of VasH_{V52} in N16961 Δ vasH does not restore the antimicrobial property of *V. cholerae*. The T6SS-mediated *E. coli* killing property was tested with V52 Δ vasH and N16961 Δ vasH carrying plasmid pVasH_{V52}::myc. Each *V. cholerae* strain was mixed with rifampin-resistant *E. coli* MG1655 at a 10:1 ratio and spotted on LB agar plates in the presence or absence of 0.1% arabinose. After 4 h of incubation at 37°C, bacterial cells were recovered from the plates, and serial dilutions were spotted on LB plates supplemented with rifampin to select for surviving *E. coli*. The experiment was done in duplicate. α , anti.

ferences in this domain are synonymous, except in the non-O1/non-O139 serogroup strain TMA_21, in which a lysine residue is found at position 321 instead of a glutamine. Since lysine is conserved at this position in the regulatory domain of the related AtoC response regulator (VC1522) in all *V. cholerae* strains, a lysine at this position is unlikely to have an effect on the activity of this domain. Thus, the conserved nature of the activator and HTH domains suggests selection against nonsynonymous mutations while the polymorphic regulatory domain with nonsynonymous substitutions may contribute to complex regulation patterns of VasH in different *V. cholerae* strains.

We previously demonstrated that episomal expression of VasH complements a *vasH*-null mutation in V52 in regard to Hcp and VasX synthesis and secretion (26). The observation that El Tor strains do not express the T6SS under laboratory conditions led us to investigate whether functional VasH of *V. cholerae* strain V52 (VasH_{V52}) can activate the T6SS of pandemic strain N16961 when episomally expressed from an

arabinose-inducible promoter. Myc epitope-tagged *vasH*_{V52} was cloned into the expression vector pBAD24 (pVasH::myc) under the control of the arabinose-inducible promoter and transformed into the N16961 Δ vasH (36) and N16961 Δ vasK strains to test for the ability of VasH_{V52} to complement virulence and protein secretion, respectively. The isogenic *vasK* deletion mutant was used as a secretion-null control since the inner membrane protein VasK is essential for T6SS-dependent protein secretion (36, 37). N16961 Δ vasH/pVasH_{V52}::myc and N16961 Δ vasK/pVasH_{V52}::myc were grown to mid-logarithmic phase of growth in the presence or absence of arabinose, and both pellet and culture supernatant fractions were subjected to Western blotting and probed with anti-VasX, anti-Myc, anti-Hcp, anti-Bla (β -lactamase, lysis control), and anti-DnaK (loading control) antibodies. As shown in Fig. 2A, expression of *vasH*_{V52} in *trans* established synthesis but not export of VasX and Hcp in N16961.

We proceeded to test if episomally expressed *vasH*_{V52} leads to N16961 virulence toward *D. discoideum* and *E. coli*. The

FIG. 3. The El Tor strain N16961 encodes a functional VasH. (A) Western blot depicting complementation of a *vasH*-null mutation with VasH_{N16961} in V52 and N16961. V52 Δ vasH and N16961 Δ vasH strains were complemented with either VasH_{V52} or VasH_{N16961} and grown until mid-logarithmic phase in the presence or absence of arabinose. Both pellet and culture supernatant fractions were subjected to SDS-PAGE and Western blot analysis using the indicated antibodies to detect T6SS-dependent protein expression and secretion. (B) VasH_{N16961} activates transcription of T6SS genes to wild-type level. V52 Δ vasH was transformed with pVasH_{V52}::myc or pVasH_{N16961}::myc, and cells were grown in the absence or presence of arabinose. The mRNA from each treatment was treated with DNase, reverse transcribed, and subjected to RT-qPCR to examine differences in transcription. The experiment was performed in triplicates. (C) Plaque assay showing that VasH_{N16961} and VasH_{V52} are equally functional. V52 Δ vasH and N16961 Δ vasH strains were complemented with pVasH_{V52}::myc or pVasH_{N16961}::myc and tested for their ability to mediate resistance toward *D. discoideum* grazing. A total of 100 μ l of overnight culture and approximately 1×10^3 AX3 cells were spread on SM/5 plates with or without arabinose. Plates were incubated at 22°C for 3 days before pictures were taken.

plaque assay using *D. discoideum* amoebae involves plating the bacterial strain of interest together with amoebae on nutrient agar plates that support only bacterial growth. In order for the amoebae to survive on agar plates, they must obtain nutrients from phagocytosed bacteria. This amoeboid grazing behavior on bacteria results in the formation of plaques, clear zones in the bacterial lawn that are devoid of bacteria. Virulent bacteria kill *D. discoideum* and prevent plaque formation. The results of the plaque assay showed that *vasH*_{V52} expression complements virulence of the V52 Δ vasH strain but in N16961 Δ vasH does not lead to virulence toward *D. discoideum* (Fig. 2B).

We previously demonstrated that V52 uses its T6SS to kill Gram-negative bacteria such as *E. coli* (24). As shown in Fig. 2C, T6SS-mediated killing requires VasH since the V52 Δ vasH strain does not kill *E. coli* unless complemented with episomal *vasH*. To examine if expression of VasH_{V52} mediates T6SS-mediated bactericidal activity in N16961, we performed an *E. coli* killing assay with N16961 Δ vasH/pVasH_{V52}::myc. Expression of VasH_{V52} in the N16961 Δ vasH strain did not confer T6SS-mediated killing of *E. coli*. In conclusion, VasH_{V52} is

essential for T6SS-mediated secretion of Hcp and VasX (26) and bacterial killing by V52 but is not sufficient to enable N16961 to secrete Hcp and VasX or kill *D. discoideum* or *E. coli*.

The El Tor strain N16961 carries a functional VasH gene. To further investigate how the T6SS is regulated in different *V. cholerae* strains, we compared the VasH polypeptide sequence from strains N16961 (VasH_{N16961}) and V52 (VasH_{V52}). VasH_{N16961} differs from VasH_{V52} by two amino acids: a histidine instead of an aspartic acid at position 116 (H116D) in the regulatory domain and a threonine instead of an alanine at position 449 (T449A) in the linker region between the putative activator domain and HTH domain. Many *V. cholerae* genomes encode VasH sequences identical to the N16961 sequence (27), but the biological relevance of this polymorphism is currently not understood. To determine whether the polymorphic nature of VasH contributes to the differential regulation of the T6SS in different genetic *V. cholerae* backgrounds, we tested if expression of VasH containing H116D and T449A (VasH_{N16961}) affects VasH function. As shown in Fig. 3A,

FIG. 4. VasH requires Walker motifs. (A) Western blot analysis comparing Hcp and VasX expression by V52 $\Delta vasH$ strains complemented by wild-type and Walker motif mutants of VasH_{V52}. Bacterial cultures were grown to mid-logarithmic phase of growth in the presence or absence of 0.1% arabinose, and pellet fractions were subjected to SDS-PAGE followed by Western blot analysis using anti-VasX, anti-DnaK (loading control), anti-Myc, and anti-Hcp antibodies. (B) Chromatographs confirming alanine substitutions in the Walker motifs (h indicates hydrophobic residues, x indicates any residue). The codon (AAA) encoding lysine (K) was changed to GCA to encode alanine (A) in the Walker A motif. Aspartic acid (D), encoded by GAT in the Walker B motif, was changed to alanine (GCT). (C) Plaque assay demonstrating that Walker mutants of VasH_{V52} are unable to restore virulence toward *D. discoideum*. Overnight bacterial culture volumes of 100 μ l were mixed with 1×10^3 amoebae on SM/5 nutrient agar plate with or without 0.1% arabinose and incubated for 3 days at 22°C. This figure represents three independent experiments. (D) Walker mutants are unable to initiate transcription of the T6SS genes. Total mRNA was extracted from mid-logarithmically growing cells. Equal amounts of RNA were treated with DNase, reverse transcribed into cDNA, and analyzed with RT-qPCR to determine the transcriptional profile of the T6SS genes: VCA0017 (*hcp-2*), VCA0018 (*vgrG-2*), and VCA0020 (*vasX*). Fold change in transcription is plotted on a log₁₀ scale. Each experiment was carried out in triplicates. WT, wild type.

VasH_{N16961} complemented the *vasH*-null mutation in a V52 background with respect to Hcp/VasX synthesis and secretion; complementation of the *vasH*-null mutation in an N16961 background restored Hcp synthesis but not secretion. VasH_{N16961} activated transcription of T6SS genes to wild-type levels (Fig. 3B), and only complemented V52 $\Delta vasH$ regained virulence toward *D. discoideum* (Fig. 3C). Thus, even though N16961 possesses a fully functional VasH that complements a *vasH*-null mutation in V52, overexpression of VasH is not sufficient to activate T6SS-mediated virulence in N16961.

Walker and HTH motifs are required for production of T6SS genes. Our bioinformatic analysis suggested the presence of three domains in the VasH polypeptide: an N-terminal activator domain, a C-terminal DNA-binding domain, and a central domain with two Walker motifs. In order to test if

VasH requires the Walker motifs to act as a σ^{54} -activator protein, two amino acids of the central catalytic domain were substituted to impair the ability of the Walker motifs to bind and hydrolyze ATP (15, 18): lysine-227 was exchanged for alanine [VasH(K227A)] in the Walker A motif, and aspartic acid-292 was exchanged for alanine [VasH(D292A)] in the Walker B motif (Fig. 4B). In addition, truncated VasH proteins lacking the C-terminal HTH motif (VasH_{V52- Δ HTH}) or the putative N-terminal regulator domain (VasH_{V52- Δ N}) were tested for their ability to activate the *V. cholerae* T6SS (Fig. 5). In order to test the ability of the mutant versions to complement a V52 *vasH*-null mutation, V52 $\Delta vasH$ was transformed with plasmid pBAD24 expressing VasH_{V52::myc} (wild type), VasH(K227A)_{V52} (Walker A mutant), VasH(D292A)_{V52} (Walker B mutant), VasH_{V52- Δ N} (regulatory domain deletion),

FIG. 5. VasH requires its C terminus. (A) Western blot depicting Hcp and VasX pellet fractions of V52 $\Delta vasH$ carrying truncated VasH (*VasH_{V52-ΔN}* and *VasH_{V52-ΔHTH}*). Pellet fractions from mid-logarithmic bacterial cultures were subjected to SDS-PAGE, followed by Western blotting with anti-VasX, anti-DnaK, anti-Myc, and anti-Hcp antibodies. (B) Plaque assay using V52 $\Delta vasH$ /p*VasH_{V52-ΔN}* and p*VasH_{V52-ΔHTH}*. A total of 100 μ l of overnight bacterial culture was spread onto SM/5 nutrient agar plates (with or without 0.1% arabinose) together with approximately 1×10^5 *D. discoideum* cells. Images were taken after 3 days of incubation at 22°C. (C) RT-qPCR data showing the ability of *VasH_{V52-ΔN}* and *VasH_{V52-ΔHTH}* to initiate T6SS gene transcription. V52 $\Delta vasH$ /p*VasH_{V52-ΔN}* and V52 $\Delta vasH$ /p*VasH_{V52-ΔHTH}* were grown to mid-logarithmic phase in the presence or absence of arabinose. Total mRNA extracted from each sample was treated with DNase, followed by reverse transcription and RT-qPCR analysis against VCA0017 (*hcp-2*), VCA0018 (*vgrG-2*), and VCA0020 (*vasX*). Fold increases of T6SS genes are plotted in log₁₀ scale on the y axis. Each experiment was done in triplicates.

or *VasH_{V52-ΔHTH}* (DNA-binding motif deletion). The protein synthesis profiles were analyzed by Western blotting using antibodies against T6SS proteins as done before (Fig. 4A and 5A). Expression of wild-type *VasH_{V52::myc}* restored Hcp and VasX synthesis in V52 $\Delta vasH$ (Fig. 4A). Although the Walker motif mutants of VasH were expressed in similar quantities compared to wild-type VasH, both mutant versions (Fig. 4B) failed to complement the *vasH*-null mutation (Fig. 4A and D). *VasH_{V52-ΔHTH}* (54 kDa) was unable to restore Hcp and VasX synthesis when episomally expressed from pBAD24 (Fig. 5A). *VasH(K227A)_{V52}*, *VasH(D292A)_{V52}*, and *VasH_{V52-ΔHTH}* failed to activate transcription of VCA0017 (*hcp-2*), VCA0018 (*vgrG-2*), and VCA0020 (*vasX*) (Fig. 4D and 5C). In contrast, deletion of the N-terminal regulatory domain did not impair VasH function under these experimental conditions (Fig. 5). To determine if the true phenotype of this mutant was masked by elevated cytoplasmic VasH levels due to episomal overexpression, we lowered the expression levels of wild-type VasH and the N-terminal deletion mutant to assess their effect on Hcp production more accurately. Semiquantitative analysis of band intensities of VasH and Hcp in Western blots demonstrated that limited synthesis of the N-terminal deletion mutant of VasH produced ~5-fold less Hcp than Hcp levels in bacteria producing wild-type VasH (Fig. 6). Thus, the N-terminal regulatory domain of VasH

does not appear to function as an intramolecular inhibitor but rather as a positive regulator.

VasH mediates virulence toward *D. discoideum*. We tested whether the mutational constructs affected virulence of *V. cholerae* toward *D. discoideum*. No plaques were formed when *VasH_{V52::myc}* was expressed in the presence of arabinose, whereas the Walker mutants *VasH(K227A)_{V52}* and *VasH(D292A)_{V52}* were unable to complement the *vasH*-null mutation as amoebae formed plaques on plates supplemented with arabinose (Fig. 4C). Thus, lysine-227 in Walker A and aspartic acid-292 in Walker B are required for VasH-mediated control of the T6SS in *V. cholerae*. *VasH_{V52-ΔHTH}* also failed to complement the virulence defect of V52 $\Delta vasH$ in a plaque assay (Fig. 5B, right panels); however, the N-terminal deletion mutant restored wild-type virulence (Fig. 5B, left panels). In conclusion, VasH-mediated virulence of *V. cholerae* strain V52 toward *D. discoideum* correlates with VasH-mediated activation of transcription, protein synthesis, and secretion (26).

Mutation of Walker and HTH motifs precludes transcription of T6SS genes. To address whether the VasH mutations affect transcription, we performed a semiquantitative real-time PCR (RT-qPCR) experiment to establish the transcriptional profiles of select T6SS genes. Transcript levels in mid-logarithmically growing bacteria in the presence or absence of arabinose were standardized using the outer membrane gene

FIG. 6. Deletion of the N-terminal regulatory domain of VasH affects Hcp levels. Bacterial cultures were grown to late logarithmic stage in the presence of decreasing amounts of arabinose. Pellet samples were subjected to SDS-PAGE, and Western blotting was performed using anti-DnaK (loading control), anti-Myc (VasH), and anti-Hcp antibodies. MW, molecular weight in thousands.

ompW, whose expression is unaffected by arabinose (30). In the presence of arabinose, episomal *VasH::myc* increased the transcription of *hcp-2* (VCA0017), *vgrG-2* (VCA0018), and *vasX* (VCA0020) in *V52 ΔvasH* ~250-, ~70-, and ~10-fold, respectively (Fig. 4D). The staggered transcript profiles were consistent in three experimental data sets. The Walker A [VasH(K227A)_{V52}] and Walker B [VasH(D292A)_{V52}] mutants did not activate transcription of the same T6SS genes (Fig. 4D) in the *V52 ΔvasH* strain, demonstrating an essential role for the central domain of the σ^{54} activator VasH. We speculate that ATP hydrolysis activity of the central domain provides the energy required to change σ^{54} -RNAP to the open conformation, as has been demonstrated for other σ^{54} activators (12, 38).

Similar to the Walker mutants of VasH, *VasH_{V52-ΔHTH}* was unable to induce transcription of T6SS genes in *V52 ΔvasH* (Fig. 5C). Removal of the HTH domain from *VasH_{V52}* rendered the protein nonfunctional. DNA binding is essential for VasH function, which is in agreement with the findings of Bernard et al. that recombinant VasH binds to T6SS promoters in electrophoretic mobility shift assays (EMSAs) (2). Conversely, high intracellular levels of the N-terminal VasH mutant (*VasH_{V52-ΔN}*) demonstrated activities similar to wild-type VasH (Fig. 5). Upon lowering *VasH_{V52-ΔN}*, Hcp levels dropped disproportionately in comparison to bacteria expressing wild-type VasH (Fig. 6), suggesting that the N-terminal domain may act as a positive intramolecular activator.

Nonfunctional VasH mutants compete with wild-type VasH. Walker motifs and the DNA-binding domain provide essential functions for VasH since Walker mutants [VasH(K227A)_{V52} and VasH(D292A)_{V52}] and *VasH_{V52-ΔHTH}* are unable to activate T6SS gene expression in *V52 ΔvasH*. We tested whether nonfunctional VasH mutants were able to compete with wild-type VasH (encoded on the chromosome) for the limited number of σ^{54} -containing RNA polymerase holoenzyme complexes ($E\sigma^{54}$) with which the activator can interact on the chromosome. Wild-type *V52* was transformed with *pVasH_{V52-K227A::myc}*, *pVasH_{V52-D292A::myc}*, or *pVasH_{V52-ΔHTH::myc}* to assess if expression in a wild-type background inhibited T6SS activity. The intensities of the Hcp and *VasX* bands in the pellet fraction decreased upon induction of the Walker motif mutants [VasH(K227A)_{V52::myc} and VasH(D292A)_{V52::myc}] in the presence of arabinose (Fig. 7A). The sample sizes were normalized based on OD₆₀₀ measurements, as indicated by the equal inten-

sities of DnaK bands. The effects of expression of the VasH variants were also assessed in the *D. discoideum* plaque assay. As shown in Fig. 7B, no plaques formed in the absence of arabinose because *V52* intrinsically resists amoeboid grazing. Episomal expression of *VasH_{V52-ΔHTH}* in *V52* did not interfere with protein synthesis and virulence toward *D. discoideum* (Fig. 7A and B). In contrast, expression of the Walker mutants of *VasH_{V52}* allowed *D. discoideum* to form plaques in the bacterial lawn (Fig. 7B). In summary, VasH without its DNA binding domain did not interfere with wild-type activity, suggesting that DNA binding precedes interaction with $E\sigma^{54}$ -promoter complexes. Expression of VasH with mutations in its Walker motifs competed with wild-type VasH for a limited number of $E\sigma^{54}$ promoter complexes. We hypothesize that Walker motif mutants act as dominant negatives by blocking the activity of wild-type VasH.

DISCUSSION

Bacteria utilize protein secretion systems for a variety of cellular processes, including signal transduction, exchange of genetic material, nutrient acquisition, and secretion of virulence factors (11). The most recently discovered secretion system in Gram-negative bacteria, the T6SS, is present in many plant and animal pathogens, and its virulence potential has been assessed in a number of different host systems. The purpose of this study was to determine how the T6SS is regulated in *V. cholerae* because in some strains, like the O37 serogroup strain *V52*, the T6SS is constitutively active, while the El Tor strain N16961 suppresses this system under laboratory conditions. We focused on the regulatory T6SS component, VasH, and mutated each of its postulated three functional domains to determine its role in endemic and pandemic *V. cholerae* strains.

We originally demonstrated that VasH is essential for T6SS-mediated virulence toward *D. discoideum* (36). Bioinformatic analyses suggested that *vasH* encodes a σ^{54} activator composed of three functional domains, typical for such a molecule. The first domain we analyzed was the putative N-terminal regulatory domain that exhibits homology to GAF domains. Deletion of the N-terminal 190 amino acids, resulting in *VasH_{V52-ΔN}*, was tolerated and did not significantly affect T6SS activation when *vasH_{V52-ΔN}* was expressed from the high-copy-number plasmid *pBAD24* (14). However, when intracellular *VasH_{V52-ΔN}* levels were lowered, we noticed a more severe

FIG. 7. Defined VasH mutants have a dominant negative effect. (A) Western blot depicting the effect of Walker motif mutants on Hcp synthesis. Wild-type V52 strains carrying nonfunctional VasH (VasH_{V52-K227A}, VasH_{V52-D292A}, and VasH_{V52- Δ HTH}) in pBAD24 were grown to mid-logarithmic phase with or without 0.1% arabinose and separated into pellet and supernatant fractions. Both fractions were subjected to SDS-PAGE and Western blotting with the antibodies shown. (B) Episomally expressed VasH Walker mutants compete with the endogenous VasH (VCA0117). Approximately 1×10^3 amoebae were plated onto SM/5 nutrient agar plates with 100 μ l of overnight bacterial culture and incubated for 3 days at 22°C. Results are representative of three independent experiments.

drop in Hcp levels than in bacteria that express wild-type VasH. Thus, it appears unlikely that the N-terminal portion of VasH acts as an intramolecular inhibitor, in which case the removal of this domain should have resulted in increased transcriptional activation, as has been proposed for the bEBP DctD from *Rhizobium meliloti* (13). Our data suggest that the N-terminal domain functions as an activator of the central domain similar to NtrC (nitrogen-regulatory protein C) from *Klebsiella pneumoniae* (8).

The removal of the helix-turn-helix (HTH) motif of some bEBPs does not affect the ability to induce gene expression when the mutant protein is present in high quantities (9, 19, 31). In the case of VasH_{V52}, deletion of the HTH motif from the C terminus resulted in a nonfunctional protein that was unable to rescue T6SS gene expression and Hcp synthesis, compete with wild-type VasH_{V52}, or restore virulence of V52 Δ vasH toward *D. discoideum*. A well-studied σ^{54} activator, NtrC from *Salmonella enterica* serovar Typhimurium, requires binding to the enhancer sequence through the HTH motif for efficient formation of ATPase-active oligomers (6). It is possible that VasH follows a pattern similar to that of NtrC in that DNA binding is required for efficient activation of the protein and/or for its activity, which cannot be compensated by increased protein-protein interaction due to overexpression.

In the *V. cholerae* O1 strain A1552, Hcp synthesis is growth phase dependent and involves the quorum-sensing regulators HapR and LuxO, RpoN, and the CRP global transcriptional regulatory complex (16). However, we were unable to detect growth-dependent production of Hcp in the El Tor strains N16961 and C6706; both strains failed to produce Hcp even during late stationary growth phase (data not shown). The two promoters of the auxiliary T6SS promoters 5' to *hcp-1* (VCA1415) and *hcp-2* (VCA0017) contain putative σ^{54} binding sequences (48) that are conserved in pandemic strains.

RT-qPCR analysis of strains overexpressing *vasH* confirmed that VasH upregulates the transcription of the important T6SS genes *hcp-2*, *vgrG-2*, and *vasX*. Binding of VasH to the *hcp* promoters was recently demonstrated in EMSAs (2), suggesting that VasH directly affects T6SS gene transcription. We suspect that the integration host factor (IHF), for which binding sites are predicted in both *hcp* promoters (48), plays an important role in the activation of *V. cholerae* T6SS. Stonehouse et al. demonstrated that IHF and histone-like nucleoid structuring proteins (H-NS; encoded by VC1130) coordinate transcription from the *ctxAB* (cholera toxin) and *tcpA* (toxin-coregulated pili) promoters in *V. cholerae* (45). H-NS is a small protein that binds to AT-rich regions of promoter sequences to silence gene expression in response to a variety of environmental signals (32). H-NS represses *ctxAB* and *tcpA* expression by binding directly to multiple sites, including the IHF and ToxT binding sites in the *tcpA-F*, *toxT*, and *ctxAB* promoters of the ToxR regulon (45). The transcriptional regulator HlyU upregulates virulence genes such as *hlyA* (hemolysin) (48), *rtx* (repeats in toxin) (21), and *hcp* (48). In addition, HlyU upregulates expression of the RTX toxin by alleviating H-NS repression bound to multiple regions of the *rtx* operon promoter (22, 48). We hypothesize that H-NS and HlyU play a role in regulating the *V. cholerae* T6SS. Because the putative IHF binding sequences in the *hcp* promoters are AT rich, H-NS may bind at the IHF binding site to inhibit IHF binding, as is the case for the regulation of CT and TCP. Moreover, a putative H-NS binding consensus motif (TCGATAAATT in an AT-rich region) (45) can be found in both *hcp* promoters upstream of the transcriptional start sites. HlyU may assist in releasing H-NS binding by yet to be identified mechanisms, allowing efficient IHF binding and subsequent transcription initiation by VasH.

While *V. cholerae* V52 and some environmental strains con-

stitutively express the T6SS (36, 51; also Provenzano, personal communication), El Tor strains such as N16961 appear to selectively use the T6SS and as a result do not express this system under laboratory conditions (27). The experiments presented here define a role for VasH in regulation of the T6SS in pandemic El Tor strains as *vasH* expression restored Hcp and VasX synthesis. However, episomal expression of VasH was unable to establish Hcp secretion in the pandemic strain N16961 and, as a consequence, T6SS-dependent virulence toward eukaryotic or prokaryotic target cells. Thus, expression of VasH only partially restores the T6SS in El Tor strains, which may produce T6SS inhibitors that function independently of VasH. The T6SS repressor TsrA is a candidate for such an inhibitor, and the interaction between VasH and TsrA needs to be examined (51). We speculate that pandemic strains suppress constitutive expression of T6SS genes to ensure that this secretion system is utilized only when it is beneficial to the organism (51). Future experiments are required to determine how VasH is involved in T6SS suppression and reactivation and whether the polymorphic nature of VasH accounts for the different utilization strategies of this virulence determinant.

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