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 the diarrheal disease 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 and inter-species competiveness. 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 (vgrGI-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 bacteriocytic 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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In a previous report, we suggested that vasH encodes an activator of the alternative sigma-54 (σ^54) factor that controls the expression of VasH and σ^54 (encoded by ptpN) 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 recombinant VasH binds the promoter of the putative N-terminal regulator domain (ψ VasH) of T6SSs, 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 (VCA0117) (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 ΔvasH 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 μg · mL^-1 ampicillin or 50 μg · mL^-1 kanamycin. E. coli strains DH5^-α and SM10 αpir were used for cloning and mating purposes, respectively. D. discoideum AX3 cells, obtained from the Dictyostelium 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.

**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
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</thead>
<tbody>
<tr>
<td>V. cholerae V52</td>
<td>O37 serogroup strain; ΔhupA ΔrpaA ΔbhvA Sm^-</td>
<td>34 Mekalanos laboratory, Harvard Medical School</td>
</tr>
<tr>
<td>V. cholerae O16961</td>
<td>El Tor O1; Sm^-</td>
<td>34 Mekalanos laboratory, Harvard Medical School</td>
</tr>
<tr>
<td>V. cholerae O395</td>
<td>Classical O1, Ogawa; Sm^-</td>
<td>34 Provenzano laboratory, University of Texas at Brownsville, TX</td>
</tr>
<tr>
<td>E. coli DH5^-α αpir</td>
<td>λ^- ph80lacZAM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(tK^- mK^-) supE44 thy1 gyrA relA1</td>
<td>Fermentas</td>
</tr>
<tr>
<td>E. coli SM10 αpir</td>
<td>thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu pir Km^-</td>
<td>Raliov laboratory, University of Alberta</td>
</tr>
<tr>
<td>E. coli GM2163</td>
<td>F^- dam-13::Tn9 (Cam^-) dcm-6 hsdR2 (tK^- mK^-) leuB6 hisG4 thi-1 ara14 lacY galK2 galT22 xylA5 mtl-1 rpsL136 (Str^-) fabA31 tss-78 glyV44 merc mcrB1</td>
<td>Stratagene</td>
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<tr>
<td>E. coli MG1655</td>
<td>F^- ΔlviC rfb-S0 phr-I</td>
<td>Taylor laboratory, University of Alberta</td>
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<tr>
<td>E. coli XL10 Gold</td>
<td>endA1 glmV44 recA1 thi-1 gyrA96 relA1 lacHc Δ(mcrA::183 Δ(mcrCB:: hsdSMR-mrr)173 Tet' [F^proAB lacP::ZAM15 Tn10(Tet') Amy Cm'])</td>
<td>14</td>
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<td>E. coli BL21*(DE3)</td>
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<td>Plasmids</td>
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<td>pBAD24</td>
<td>pBAD vector, pBR322 ori, AraC, Amp^-</td>
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<td>pWM91</td>
<td>oriR6K mobRP4 lacI pac tnp mini-Tn10Km; Km^- Amp^-</td>
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<tr>
<td>pGEM-T Easy</td>
<td>Vector for cloning PCR products; Amp^-</td>
<td>Promega</td>
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</table>

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 (vasH132) with the primer pairs VCA0117A/VCA0117B (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-VasH52::myc was digested with EcoRI and XbaI and the 5^-myc-VasH and VasH52 with a deletion of the putative N-terminal regulator domain (vasH52AN) and vasH52 with a deletion of the C-terminal helix-turn-helix (HTH) motif (vasH52MTR67) were generated in the same manner using the primer pair 5^-myc-VasH and VasH52AN and the pair 5^-myc-VasH and VasH52MTR67, respectively.

**Site-directed mutagenesis of VasH.** Amino acid substitutions were introduced into VasH using a QuiChange 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 PBAD promoter in pBAD24. Bacterial cultures were centrifuged at 14,000 × 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 μl were filtered through 0.22-μm-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 × g for 5 min at
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-VasH primary antibodies. Secondary antibodies included donkey anti-rabbit radish peroxidase (HRP) and goat anti-rabbit HRP (both diluted 1:1,000) sec-

TABLE 2. Oligonucleotide sequences

| Primer name and function | Direction | Sequence
<table>
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<td>VCA0117-A</td>
<td>F</td>
<td>GGATCCAGCAGTGATATCGACGAC</td>
</tr>
<tr>
<td>VCA0117-B</td>
<td>R</td>
<td>GCGTAAACGTTGCCAAGTGGAT</td>
</tr>
<tr>
<td>VCA0117-C</td>
<td>F</td>
<td>GTAACCATGATGAATGCGGCCATAG</td>
</tr>
<tr>
<td>VCA0117-D</td>
<td>R</td>
<td>GAGTTCATCGTGCAAGATGAT</td>
</tr>
</tbody>
</table>

& 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, 0.1, 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 × 10⁴ 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 (vasH), and VCA0117 (vasK) 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 around 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 (ΔΔCT) 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 counted after plates were incubated at 22°C for 3 days. The protocol was adapted from MacIntyre et al. (24).

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measured by serial dilution and selective growth on agar containing 50 μg·ml⁻¹ rifampin and 100 μg·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 σ⁵₄ activator domain in the center of VasH (E value, 2.2E⁻¹⁵) 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 σ⁵₄-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 3.6E⁻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 σ⁵₄ 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-X₇-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 finding by Bernard et al. that VasH binds T6SS promoters (2) suggests that VasH drives σ⁵₄-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 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.

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 σ⁵₄-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.
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 Hep 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. 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 Hep 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 D. discoideum cells were plated onto SM/5 nutrient agar plates (with or without 0.1% arabinose) with 100 μl of bacterial strains. Pictures were taken on the third day of incubation at 22°C. The T6SS-mediated E. coli killing property was tested with V52 ΔvasH and N16961 ΔvasH 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.

FIG. 2. Episomal expression of VasH_V52 induces Hep synthesis but not secretion in N16961. (A) Expression of VasH_V52::myc establishes Hep 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 ΔvasH. Approximately 1 × 10^6 mid-logarithmic D. discoideum cells were plated onto SM/5 nutrient agar plates (with or without 0.1% arabinose) with 100 μl of bacterial strains. Pictures were taken on the third day of incubation at 22°C. (C) Expression of VasH_V52 in N16961 ΔvasH does not restore the antimicrobial property of V. cholerae. The T6SS-mediated E. coli killing property was tested with V52 ΔvasH and N16961 ΔvasH 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.
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* expression complements virulence of the V52/H9004 *vasH* strain but in N16961/H9004 *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 ΔvasH strain does not kill *E. coli* unless complemented with episomal *vasH*. To examine if expression of *VasH* mediates T6SS-mediated bactericidal activity in N16961, we performed an *E. coli* killing assay with N16961/H9004 *vasH/pVasHV52::myc*. Expression of *VasH* in the N16961 ΔvasH strain did not confer T6SS-mediated killing of *E. coli*. In conclusion, *VasH* 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,
VasHN16961 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. VasHN16961 activated transcription of T6SS genes to wild-type levels (Fig. 3B), and only complemented V52/H9004 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 Walker motifs to act as a σ54-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 (D), encoded by GAT in the Walker B motif, was changed to alanine (GCT). In addition, truncated VasH proteins lacking the C-terminal HTH motif (VasH_V52-ΔHTH) or the putative N-terminal regulator domain (VasH_V52-ΔN) 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 ΔvasH 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-ΔN (regulatory domain deletion), and VasH_V52-ΔHTH (central domain deletion).
or VasHV52-\(\Delta\)HTH (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 VasHV52::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). VasHV52-\(\Delta\)myc restored Hcp and VasX synthesis in V52\(\Delta\)vasH when episomally expressed from pBAD24 (Fig. 5A). VasH(K227A)\(\Delta\)V52, VasH(D292A)\(\Delta\)V52, and VasH V52-\(\Delta\)myc were unable to complement the vasH-null mutation to initiate T6SS gene transcription. V52\(\Delta\)vasH/pVasHV52-\(\Delta\)myc and V52\(\Delta\)vasH/pVasHV52-\(\Delta\)HTH 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 log\(_{10}\) scale on the y axis. Each experiment was done in triplicates.

FIG. 5. VasH requires its C terminus. (A) Western blot depicting Hcp and VasX pellet fractions of V52\(\Delta\)vasH carrying truncated VasH (VasHV52-\(\Delta\)myc and VasHV52-\(\Delta\)HTH). 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/pVasHV52-\(\Delta\)myc and pVasHV52-\(\Delta\)HTH. A total of 100 \(\mu\)l of overnight bacterial culture was spread onto SM/5 nutrient agar plates (with or without 0.1% arabinose) together with approximately \(1 \times 10^3\) \(D.\) discoideum cells. Images were taken after 3 days of incubation at 22°C. (C) RT-qPCR data showing the ability of VasHV52-\(\Delta\)myc and VasHV52-\(\Delta\)HTH to initiate T6SS gene transcription. V52\(\Delta\)vasH/pVasHV52-\(\Delta\)myc and V52\(\Delta\)vasH/pVasHV52-\(\Delta\)HTH 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 log\(_{10}\) scale on the y axis. Each experiment was done in triplicates.

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 VasHV52::myc was expressed in the presence of arabinose, whereas the Walker mutants VasH(K227A)\(\Delta\)V52 and VasH(D292A)\(\Delta\)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. VasHV52-\(\Delta\)HTH also failed to complement the virulence defect of V52\(\Delta\)vasH in a plaque assay (Fig. 4C). In contrast, deletion of the N-terminal deletion mutant restored wild-type virulence (Fig. 4D, 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
ompW, whose expression is unaffected by arabinose (30). In the presence of arabinose, episomal VasH::myc increased the transcription of hcp-2 (VCA0017), vvgG-2 (VCA0018), and vasX (VCA0020) in V52 ∆vasH ∼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 ∆vasH strain, demonstrating an essential role for the central domain of the σ54 activator VasH. We speculate that ATP hydrolysis activity of the central domain provides the energy required to change σ54-RNAP to the open conformation, as has been demonstrated for other σ54 activators (12, 38).

Similar to the Walker mutants of VasH, VasHV52-D292A::myc and VasHV52 K227A::myc in the presence of arabinose (Fig. 7A). The sample sizes were normalized to OD600 measurements, as indicated by the equal intensities 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 VasHV52-D292A::myc 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 VasHV52 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 Erσ54 promoter complexes. Expression of VasH with mutations in its Walker motifs competed with wild-type VasH for a limited number of Erσ54 promoter complexes. We hypothesize that Walker motif mutants act as dominant negatives by blocking the activity of wild-type VasH.

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.

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 VasHV52-D292A::myc are unable to activate T6SS gene expression in V52 ∆vasH. We tested whether nonfunctional VasH mutants were able to compete with wild-type VasH (encoded on the chromosome) for the limited number of σ54 containing RNA polymerase holoenzyme complexes (Erσ54) with which the activator can interact on the chromosome. Wild-type V52 was transformed with pVasHV52, K227A::myc, pVasHV52, D292A::myc, or pVasHV52-D292A::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 intensities 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 VasHV52-D292A::myc 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 VasHV52 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 Erσ54 promoter complexes. Expression of VasH with mutations in its Walker motifs competed with wild-type VasH for a limited number of Erσ54 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 σ54 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 VasHV52-D292A::myc, was tolerated and did not significantly affect T6SS activation when vasHV52-D292A::myc was expressed from the high-copy-number plasmid pBAD24 (14). However, when intracellular VasHV52-D292A::myc levels were lowered, we noticed a more severe
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 DetD 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

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 restructuring 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*, *toxA*, 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-
stutitutively 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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