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# Response of *Vibrio cholerae* to Low-Temperature Shifts: CspV Regulation of Type VI Secretion, Biofilm Formation, and Association with Zooplankton

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**ABSTRACT**

**The ability to sense and adapt to temperature fluctuation is critical to the aquatic survival, transmission, and infectivity of *Vibrio cholerae*, the causative agent of the disease cholera. Little information is available on the physiological changes that occur when *V. cholerae* experiences temperature shifts. The genome-wide transcriptional profile of *V. cholerae* upon a shift in human body temperature (37°C) to lower temperatures, 15°C and 25°C, which mimic those found in the aquatic environment, was determined. Differentially expressed genes included those involved in the cold shock response, biofilm formation, type VI secretion, and virulence. Analysis of a mutant lacking the cold shock gene *cspV*, which was upregulated >50-fold upon a low-temperature shift, revealed that it regulates genes involved in biofilm formation and type VI secretion. CspV controls biofilm formation through modulation of the second messenger cyclic diguanylate and regulates type VI-mediated interspecies killing in a temperature-dependent manner. Furthermore, a strain lacking *cspV* had significant defects for attachment and type VI-mediated killing on the surface of the aquatic crustacean *Daphnia magna*. Collectively, these studies reveal that *cspV* is a major regulator of the temperature downshift response and plays an important role in controlling cellular processes crucial to the infectious cycle of *V. cholerae*.**

**IMPORTANCE**

**Little is known about how human pathogens respond and adapt to ever-changing parameters of natural habitats outside the human host and how environmental adaptation alters dissemination. *Vibrio cholerae*, the causative agent of the severe diarrheal disease cholera, experiences fluctuations in temperature in its natural aquatic habitats and during the infection process. Furthermore, temperature is a critical environmental signal governing the occurrence of *V. cholerae* and cholera outbreaks. In this study, we showed that *V. cholerae* reprograms its transcriptome in response to fluctuations in temperature, which results in changes to biofilm formation and type VI secretion system activation. These processes in turn impact environmental survival and the virulence potential of this pathogen.**

The facultative human pathogen *Vibrio cholerae* is found in aquatic reservoirs, such as estuaries, coastal waters, and freshwater lakes and rivers, where it is exposed to seasonal and interannual fluctuations in temperature (1–3). Studies have demonstrated a positive correlation between the occurrence of *V. cholerae* and surface water temperature in a variety of aquatic environments (4–10) and between cases of the disease cholera and elevated sea surface temperatures in areas where cholera is endemic (5, 6, 11). These aquatic habitats exhibit a temperature range of about 12°C to 30°C (5, 6), whereas the human host environment is a constant 37°C. Temperature is hypothesized to be a key signal to differentiate between host and environmental reservoirs, facilitating virulence factor expression in the human host and genes associated with environmental survival upon expulsion. Studies aimed at understanding the importance of temperature upshift in *V. cholerae* have shed light on mechanisms crucial to the survival of this pathogen inside the human host (12–14). However, less information is available regarding how *V. cholerae* survives and adapts to the downshift in temperature the pathogen experiences upon exiting the human host and entering the environment, which is crucial to the perpetuation of its infectious cycle.

Low-temperature environments impose numerous challenges to bacterial cell physiology. As temperatures decrease, the lipid composition of the cell membrane transitions from a liquid crystalline state to a rigid gel state (15, 16). Translation is impeded, as low temperatures cause poor ribosome assembly and the formation of extensive RNA secondary structures (17, 18). In *Escherichia coli*, a shift to low temperatures causes the upregulation of genes that encode proteins involved in ribosome function, helicase activity, and exoribonuclease activity, as well as four cold shock proteins (CSPs) (16). CSPs are predicted to counteract the widespread effects of RNA secondary-structure stabilization as chaperones (19, 20). The *V. cholerae* genome encodes four predicted

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cold shock genes, *cspA*, *cspV*, VCA0184, and VC1142; however, a previous study determined that only CspA and CspV were highly induced when exponentially growing *V. cholerae* cells were shifted from 37°C to lower temperatures (21). Currently, little is known about how these proteins and low-temperature shifts affect the physiology and cellular processes of this pathogen.

Biofilm formation is a cellular process that is critical to the infectious cycle and environmental survival of *V. cholerae* and is regulated by temperature (22). Biofilms are cell aggregates or surface-attached bacterial communities enclosed in an extracellular matrix. In the aquatic environment, *V. cholerae* forms biofilms on the surfaces of plankton, which greatly increases its survival and facilitates dissemination of the pathogen (23–26). Additionally, biofilm-like aggregates of *V. cholerae* have been found in the surface waters of Bangladesh in a partially dormant "conditionally viable" state (27). The extracellular matrix that surrounds the biofilm of *V. cholerae* is composed of *Vibrio* polysaccharides (VPS) (28, 29), matrix proteins RbmA, RbmC, and Bap1 (30, 31), and extracellular DNA (32). The *vps*-I (*vpsU-vpsK*) and *vps*-II (*vpsL-vpsQ*) operons are responsible for the biosynthesis of VPS and are required for mature biofilm formation (28, 29). Transcriptional regulation of biofilm genes is controlled by the activators AphA, VpsR, and VpsT and the repressor HapR. The response regulators VpsR and VpsT bind the *vps* promoters directly (33), while AphA affects biofilm formation through *vpsT* (34). Transcription of the genes that encode VPS and the matrix proteins is increased when levels of the intracellular messenger cyclic diguanylate (c-di-GMP) are high (35, 36). HapR is the master quorum-sensing regulator that represses biofilm formation at high cell density through direct binding of the *vps* regulatory region and *vpsT* (37).

The type VI secretion system (T6SS) mediates interspecies competition (38), virulence (39, 40), and natural transformation (41) in *V. cholerae*. The T6SS apparatus includes a base that spans the cell envelope, an inner tube composed of hemolysin-coregulated protein (Hcp) polymers, and an outer contractile sheath that is formed by VipA and VipB (42, 43). The genes encoding the T6SS components are organized into one large cluster (VCA0105 to VCA0124) and two auxiliary clusters (VCA0017 to VCA0022 and VC1415 to VC1421) (39, 44). Transcription of T6SS genes is positively regulated by HapR, the global regulator cyclic AMP (cAMP) receptor protein (CRP), the transcriptional regulator VasH, and the competence regulators Tfox and QstR and negatively regulated by the quorum-sensing regulator LuxO and global regulator TsrA (40, 41, 45–47). It has been reported that T6SS production is affected by temperature (48); however, it remains unclear if temperature impacts T6SS interspecies killing.

In this study, we identified *V. cholerae* genes that are temperature regulated by examination of the genome-wide transcriptional profile of *V. cholerae* upon a shift from 37°C to 15°C or 25°C. We found that the expression of genes encoding cold shock proteins, biofilm formation, T6SS, and virulence were differentially regulated upon a cold shift. We demonstrated that biofilm formation and the T6SS were affected by temperature. Mutational analysis and phenotypic characterization of the most highly induced gene, *cspV*, revealed that this cold shock gene affects biofilm formation and type VI secretion in *V. cholerae in vitro* and on the surface of the chitinous zooplankton *Daphnia magna*, revealing the mechanism by which *V. cholerae* responds to varying temperatures and how this response impacts *V. cholerae* environmental survival.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *V. cholerae* O1 El Tor A1552 was used as our wild-type strain. *E. coli* CC118λ*pir* and S17-1λ*pir* were used for cloning and conjugation, respectively. Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.2 M NaCl [pH 7.5]) was used to grow all strains. Temperature shift experiments were performed by inoculating overnight-grown cultures of *V. cholerae* in a 1:200 dilution in LB medium and incubating them at 37°C until an optical density at 600 nm ($OD_{600}$) of 0.4 was reached; cultures were then shifted to 15°C and 25°C for 1 h. All cultures were aerated by shaking at 200 rpm. When needed, ampicillin and rifampin were used at 100 μg/ml, and streptomycin was used at 50 μg/ml. Deletion constructs were generated using splicing by overlap extension PCR and cloned into pGP704-sacB28 suicide plasmids. Mutants were generated using previously published protocols (30) and verified by PCR.

**Gene expression profiling.** Microarrays used were composed of spotted 70-mer oligonucleotides representing the open reading frames of the *V. cholerae* strain N16961 genome and were printed at the University of California, Santa Cruz, CA (discussed in greater detail in reference 49). Experimental RNA samples were isolated from *V. cholerae* cells that were exposed to a cold shift. Overnight-grown cultures of *V. cholerae* were diluted 1:200 in LB medium and incubated at 37°C until cells reached to an $OD_{600}$ of 0.4; cultures were shifted to 15°C, 25°C, or maintained at 37°C for 1 h. RNA preparation and microarray hybridization and scanning were performed as described previously (49) Normalized signal ratios were obtained with locally weighted scatterplot smoothing (LOWESS) print tip normalization, using the Bioconductor packages (50) in the R environment. Differentially regulated genes were determined using three biological replicates and two technical replicates for each treatment (six data points for each spot), using the Significance Analysis of Microarrays (SAM) program (51), with a 2-fold difference in gene expression and a 3% false-discovery rate (FDR) as cutoff values.

**Expression analysis: real-time PCR.** RNA was harvested as described previously (22). Results are from two independent experiments performed in triplicate. All samples were normalized to the expression of the 16S rRNA housekeeping gene using the Pfaffl method (52). Relative expression was calculated by normalizing expression at 15°C or 25°C to that at 37°C. Statistical analysis was performed using two-tailed Student's *t* test.

**Analysis of biofilm formation.** Green fluorescent protein (GFP)-expressing *V. cholerae* cells from overnight-grown cultures were diluted into LB broth to an $OD_{600}$ of about 0.02, and then 3 ml was inoculated into glass chambers (Thermo Fisher Scientific, Waltham, MA, USA) and incubated statically at 15°C, 25°C, or 37°C. After 24 h, biofilm formation was visualized using confocal laser scanning microscopy (CLSM) with a 5-Pa laser scanning microscope (LSM) (Zeiss, Oberkochen, Germany). Three-dimensional images were reconstructed using Imaris 7.6 and analyzed using COMSTAT (53). Each experiment included three independent biological replicates, and three images were taken for each replicate.

**Determination of intracellular c-di-GMP levels.** Overnight-grown *V. cholerae* strains were diluted 1:200 and grown to an $OD_{600}$ of 4.0, at which time cultures were harvested, and the remaining culture was shifted to 15°C for 1 h and then sampled. c-di-GMP extraction was performed as described previously (22). Each c-di-GMP quantification experiment was performed with four biological replicates. Levels of c-di-GMP in cells grown at different temperatures were compared using a two-tailed Student *t* test.

**Analysis of Hcp production.** Overnight-grown *V. cholerae* strains were diluted 1:200 and grown to an $OD_{600}$ of 4.0, at which time cultures were sampled, and the remaining culture was shifted to 15°C, 25°C, or maintained at 37°C for 1 h and then sampled. Sample preparation and Western blotting were performed as previously described (40). These experiments were conducted with three biological replicates.

**Bacterial killing assay.** Killing assays were performed as described previously (44), with modifications. Bacterial strains were grown over-

night on LB plates and resuspended in LB broth. *V. cholerae* and streptomycin-resistant *E. coli* MC4100 or *Aeromonas* sp. were mixed at a 10:1 ratio, and 25 μl was spotted onto LB agar plates and incubated for 4 h. *E. coli* alone was plated and incubated as described above for comparison. All spots were harvested, serially diluted in LB agar, and plated onto LB agar plates containing 50 μg/ml of streptomycin to enumerate surviving prey cells.

**Preparation of bacterium-free and gnotobiotic *D. magna*.** Bacterium-free and gnotobiotic *D. magna* containing only one bacterial strain, *Aeromonas* (Xinb3-6, GenBank accession no. KF924766), were produced according to methods previously described. Bacterium-free animals were transferred in 80-ml sterile Aachener Daphnien medium (ADaM) and fed axenic or autoclaved *Scenedesmus obliquus* green algae. For gnotobiotic *D. magna*, the strain *Aeromonas* previously cultured in LB medium and resuspended in sterile ADaM was introduced into bacterium-free hatchlings and grown under the same environmental condition as the bacterium-free *Daphnia* animals until further experimentation.

***D. magna* attachment and T6SS killing assays.** Overnight-grown *V. cholerae* was washed twice using 1× phosphate-buffered saline (PBS). Cultures were diluted to an $OD_{600}$ of 0.02 in 5 ml ADaM and gently vortexed, and then a single *D. magna* animal was added to each culture tube and incubated statically at 25°C for 24 h. For scanning electron microscopy (SEM), *D. magna* animals were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer for 2 h, washed twice in distilled water for 10 s, and dehydrated in a graded ethanol series (30, 50, 70, and 90%). Samples were critical-point dried, sputtered with approximately 20-nm gold particles, and imaged with an FEI Quanta three-dimensional (3D) dual-beam microscope. For CFU counts, cultures were plated to enumerate the planktonic population, while the *D. magna* animals were removed, added to fresh ADaM, and then homogenized and plated to enumerate the attached population. A two-tailed Student *t* test was used to determine statistical significance. For live-*D. magna* T6SS-mediated killing assays, overnight-grown cultures of *V. cholerae* and *Aeromonas* sp. were washed twice using 1× PBS, at which time cultures were normalized to the same $OD_{600}$, mixed at a 10:1 ratio, and then diluted and added to the *D. magna* as described above. After 4 h, the *D. magna* animals were removed, added to fresh ADaM, and then homogenized and plated onto LB medium plates containing 50 μg/ml streptomycin to enumerate the surviving prey cells.

## RESULTS

**Changes to *V. cholerae* transcriptional profile upon a cold shift.** Whole-genome expression profiling revealed that a total of 595 and 254 genes were significantly differentially regulated upon being shifted from 37°C to 15°C and 25°C, respectively (see Table S1 in the supplemental material). There were 112 genes differentially regulated at both 15°C and 25°C (Table S1). Fifty genes were upregulated in similar manners upon shifts to both 15°C and 25°C, suggesting a more general role in low-temperature adaptation (Table S1). These included genes predicted to encode proteins involved in amino acid biosynthesis, energy metabolism andtransport, and binding (Table S1). Additionally, three cold shock proteins were upregulated at low temperatures (discussed in further detail below) (Table S1). Forty-eight genes were downregulated in similar manners upon shifts to both 15°C and 25°C. These genes are predicted to encode proteins involved in cellular processes, including heat shock, carbohydrate transport and binding, and transcriptional regulation (Table S1). Additionally, genes involved in pathogenesis were downregulated at low temperatures (Fig. 1). Genes differentially regulated specifically at either 15°C or 25°C postshift included those involved in biofilm formation (Fig. 1), which were upregulated at 15°C but not at 25°C (Fig. 1), and the T6SS, which were downregulated at 15°C and upregulated at 25°C. Because temperature is an important

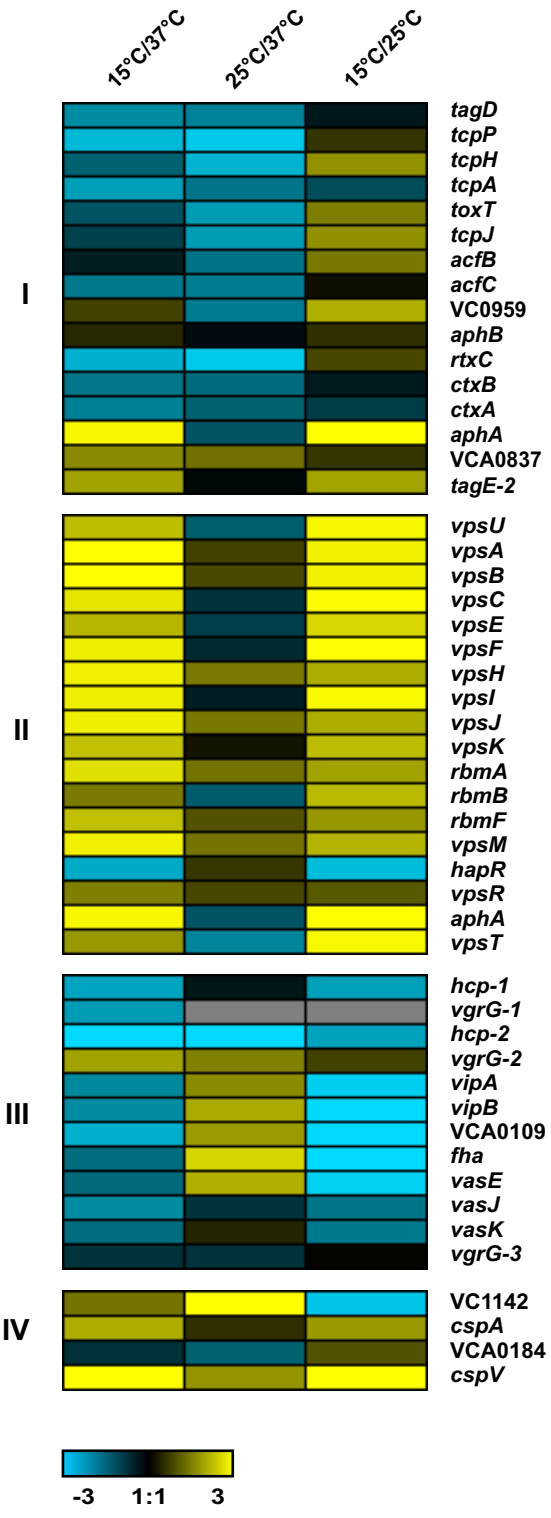


**FIG 1** The *V. cholerae* transcriptome is altered by temperature downshifts. Expression profiles of a selected set of genes in *V. cholerae* cells grown at 37°C compared to those shifted to 15°C or 25°C for 1 h. Induced expression is represented in yellow, and repressed expression is represented in blue. Differential expression of genes involved in virulence (I), biofilm formation (II), T6SS (III), and cold shock (IV) was observed upon a cold shift. The color scale is shown at the bottom. The details of the expression data are provided in Table S1 in the supplemental material.

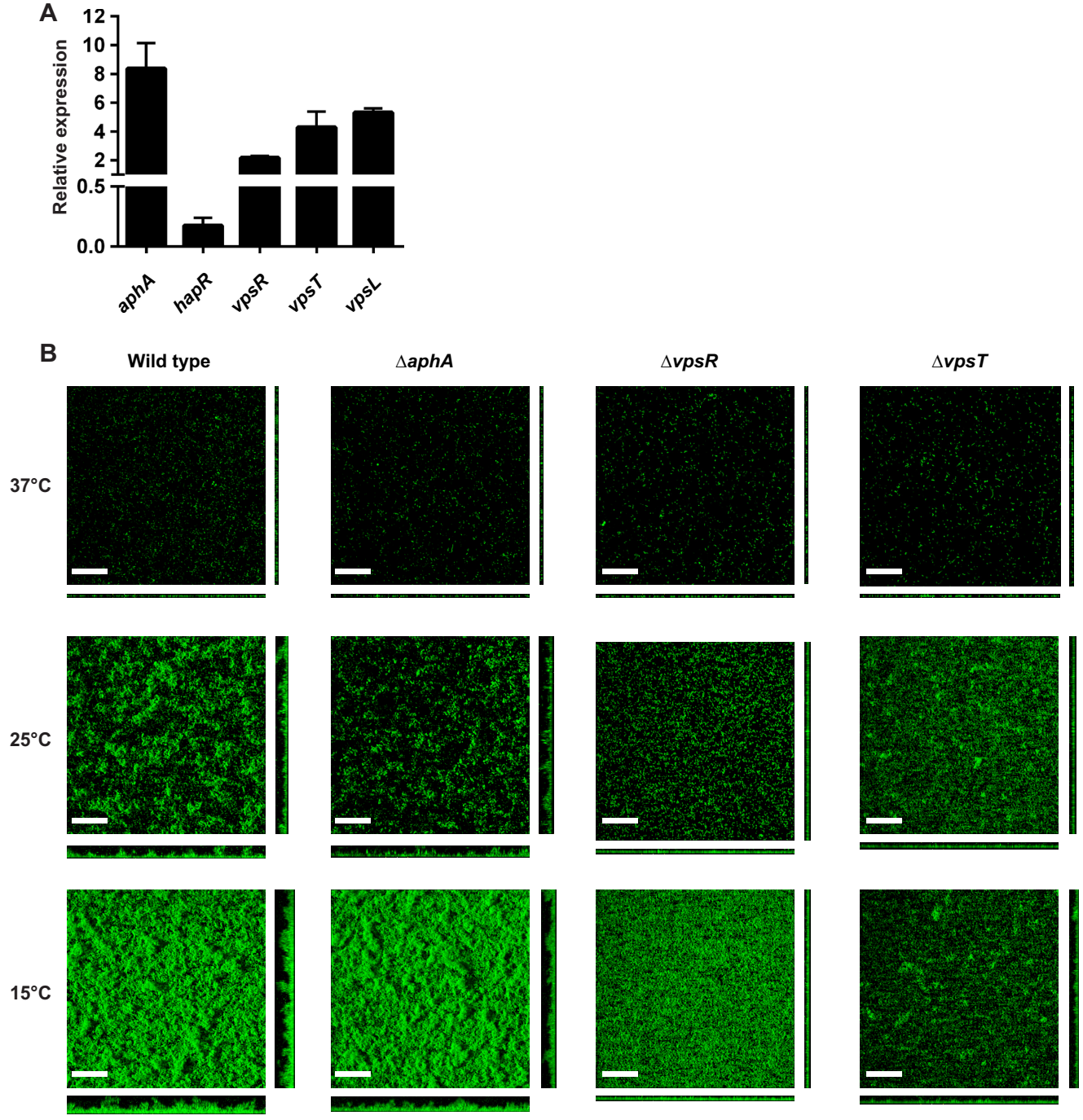


**FIG 2** Biofilm formation is modulated by temperature. (A) Analysis of biofilm gene expression in response to a low-temperature shift by qRT-PCR. Error bars indicate standard deviations of the results from three biological replicates. All genes exhibited a statistically significant difference between 15°C and 37°C ($P < 0.05$). (B) Three-dimensional biofilm structures of wild-type *V. cholerae* and the Δ*aphA*, Δ*vpsR*, and Δ*vpsT* mutants formed at 37°C, 25°C, and 15°C after 24 h of incubation. Images shown are from one representative experiment of three independent experiments. The scale bars represent 40 μm.

signal that can be used by *V. cholerae* to distinguish between the human host and the environment, we were interested in genes that might affect the behavior of *V. cholerae* upon its transition from the human host into the aquatic environment. To this end, we further examined the effect that temperature has on biofilm formation and the T6SS and tested if the CSPs affect these processes.

**Regulation of biofilm formation by low temperature.** The expression of genes encoding major biofilm regulators, *aphA*, *vpsR*, and *vpsT*, and structural components, *vps*-I, *vps*-II, and *rbmA*, were upregulated upon a shift from 37°C to 15°C, while the gene encoding a negative regulator of biofilm formation, *hapR*, was downregulated (Fig. 1; see also Table S1 in the supplemental material). In agreement with the whole-genome expression analysis, reverse transcription-quantitative PCR (qRT-PCR) analysis re-

**TABLE 1** COMSTAT analysis of biofilms formed by mutants lacking major biofilm regulators[a]

| Strain/ genotype | Temp (°C) | Biomass ($\mu m^3/\mu m^2$) | Thickness ($\mu m$) | | Roughness coefficient |
|---|---|---|---|---|---|
| | | | Avg | Maximum | |
| WT | 37 | 0.22 (0.02) | 0.30 (0.09) | 5.57 (1.02) | 1.83 (0.04) |
| | 25 | 1.64 (0.25) | 1.89 (0.15) | 15.55 (1.02) | 1.19 (0.05) |
| | 15 | 6.64 (0.23) | 7.33 (0.24) | 22.00 (2.33) | 0.43 (0.02) |
| Δ*aphA* | 37 | 0.12 (0.02) | 0.13 (0.02) | 7.63 (4.06) | 1.92 (0.02) |
| | 25 | 2.71 (0.28) | 3.35 (0.12) | 18.77 (3.09) | 0.78 (0.06) |
| | 15 | 7.41 (0.37) | 7.73 (0.42) | 23.17 (1.02) | 0.40 (0.01) |
| Δ*vpsR* | 37 | 0.17 (0.03) | 0.19 (0.02) | 5.87 (0.51) | 1.89 (0.02) |
| | 25 | 1.17 (0.18) | 1.22 (0.07) | 6.45 (1.02) | 1.30 (0.05) |
| | 15 | 3.25 (0.28) | 3.09 (0.31) | 6.16 (0.88) | 0.30 (0.05) |
| Δ*vpsT* | 37 | 0.16 (0.01) | 0.17 (0.03) | 5.28 (0.88) | 1.89 (0.01) |
| | 25 | 0.87 (0.47) | 1.17 (0.47) | 7.33 (0.51) | 1.40 (0.25) |
| | 15 | 2.37 (0.36) | 2.62 (0.27) | 9.68 (0.88) | 0.54 (0.06) |

[a] Biofilms were grown at 15°C, 25°C, and 37°C in static chambers in LB medium for 24 h. Values in parentheses are standard deviations.

vealed that *aphA*, *vpsL*, *vpsR*, and *vpsT* mRNA message abundance was increased 8.3-, 2.2-, 4.3-, and 5.3-fold, respectively, while *hapR* message abundance was decreased 7.1-fold 1 h after a shift from 37°C to 15°C (Fig. 2A). To determine if the major biofilm regulators were responsible for modulating biofilm gene expression at low temperatures, Δ*aphA*, Δ*vpsR*, and Δ*vpsT* mutants were grown at 15°C, 25°C, and 37°C in static chambers for 24 h, and biofilms were imaged and compared to wild-type *V. cholerae*. As we showed previously (22), biofilm formation is enhanced at 15°C and 25°C compared to that at 37°C (Fig. 2B and Table 1). However, the Δ*vpsR* and Δ*vpsT* mutants exhibit markedly lower biomass and thickness than the wild type at both 15°C and 25°C (Fig. 2 and Table 1). The Δ*aphA* mutant exhibited a small but reproducible decrease in biomass and thickness (Fig. 2 and Table 1) at low temperatures. These results suggest that temperature affects biofilm formation through modulation of the known biofilm regulators VpsR, VpsT, and AphA.

**Regulation of the T6SS by low temperature.** The expression of genes involved in the biogenesis of type VI secretion was downregulated at 15°C and upregulated at 25°C. qRT-PCR analysis confirmed that *hcp* mRNA message abundance was decreased nearly 1.5-fold 1 h after the shift to 15°C and increased 6.0-fold 1 h after the shift to 25°C (Fig. 3A). We compared levels of Hcp protein production and secretion in cells before (37°C) and 1 h after (15°C or 25°C) the low-temperature shift. We found that Hcp production was very low, and no secretion was observed in cells grown at 15°C; however, Hcp production and secretion were increased in cells grown at 25°C (Fig. 3B). Next, we performed T6SS-mediated killing assays at 15, 25, and 37°C using *E. coli* as a prey strain. We observed a 5-fold reduction in the number of CFU of *E. coli* recovered after incubation with wild-type *V. cholerae* at 37°C (Fig. 3C) and a 16-fold reduction when incubated with wild-type *V. cholerae* at 25°C (Fig. 3C). However, we saw no significant decrease (1.1-fold) in the number of *E. coli* CFU recovered when cells were incubated with *V. cholerae* at 15°C, indicating that under the conditions tested, wild-type *V. cholerae* does not use its T6SS to kill *E. coli* when incubated at 15°C (Fig. 3C). Collectively, these results showed that T6SS production and type VI-mediated killing are strongly modulated by temperature.

**Expression of cold shock genes.** Because low temperatures dramatically affect both biofilm formation and the T6SS, we hypothesized that the CSPs may be important for these cellular processes. To test this hypothesis, we first determined which cold shock genes were upregulated upon the shift to low temperature in *V. cholerae*. At 15°C, the message abundance of *cspV*, *cspA*, and VC1142 was increased 60.9-, 21.9-, and 2.5-fold, respectively (Fig. 4A); however, there was no significant difference for

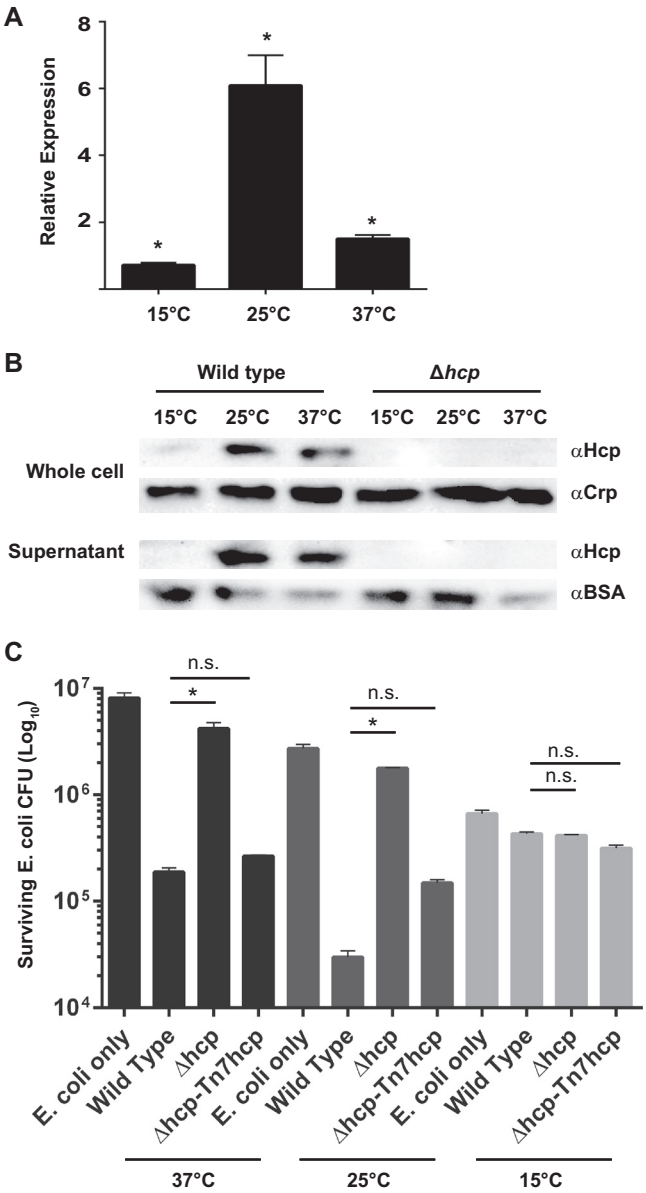


**FIG 3** The T6SS is modulated by temperature. (A) Analysis of *hcp* gene expression by qRT-PCR. Error bars indicate standard deviations of the results from three biological replicates ($P < 0.05$). (B) Hcp production levels by Western blot analysis before (at 37°C) and 1 h after (15°C or 25°C) a low-temperature shift. BSA, bovine serum albumin control; α, anti. (C) T6SS-mediated killing of *E. coli* at 37°C, 25°C, and 15°C. Error bars indicate standard deviations of the results from three biological replicates. *, $P < 0.05$; n.s., not significantly different ($P > 0.05$).

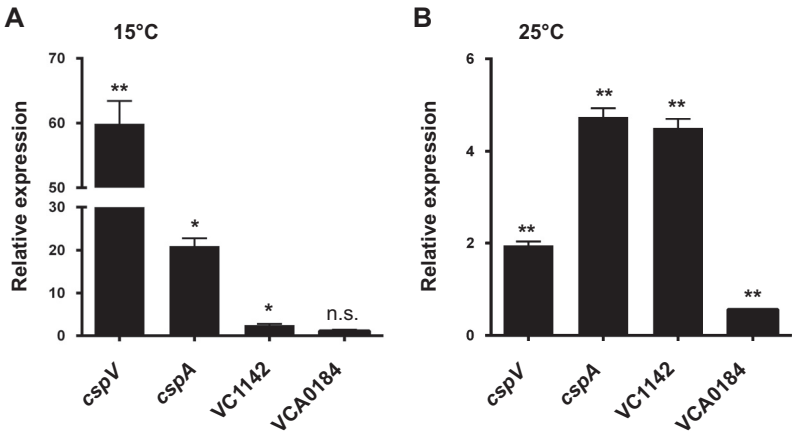


**FIG 4** Expression of three cold shock genes is differentially regulated by temperature. Analysis of cold shock gene expression in response to a shift from 37°C to 15°C (A) or 25°C (B) by qRT-PCR. Error bars indicate standard deviations of the results from three biological replicates. *, $P < 0.05$; **, $P < 0.005$; n.s., not significantly different ($P > 0.05$).

VCA0184 (Fig. 4A). At 25°C, the message abundance of *cspV*, *cspA*, and VC1142 was increased 2.0-, 4.7-, and 4.5-fold, respectively (Fig. 4B), while VCA0184 was decreased 1.8-fold (Fig. 4B). Because *cspV* was the most highly expressed gene at 15°C, the temperature at which we observed the most dramatic effect on biofilm formation and the T6SS, we further studied *cspV* to see if it plays a role in these processes.

**CspV regulates biofilm gene expression, biofilm formation, and cellular c-di-GMP levels.** To determine if *cspV* plays a role in biofilm formation at low temperatures, we first used qRT-PCR to compare the mRNA message abundances of biofilm genes in the Δ*cspV* mutant and the wild type. The Δ*cspV* mutant exhibited a significant decrease in mRNA message abundance of the positive biofilm regulators *vpsR* and *vpsT* and biofilm matrix components *vpsL* and *rbmA*, as well as an increase in that of the phosphodiesterase gene *rocS*, which negatively regulates biofilm formation through the degradation of c-di-GMP (Fig. 5A). Next, we examined biofilm formation directly in a strain lacking *cspV* during growth at 15°C, 25°C, and 37°C. When grown at 15°C, the *cspV* mutant showed 1.8-, 2.4-, and 3.7-fold decreases in biomass, average thickness, and maximum thickness, respectively, compared with the wild type (Fig. 5B and Table 2). At 25°C, the Δ*cspV* mutant showed 1.1-, 1.3-, and 1.3-fold decreases in biofilm biomass, average thickness, and maximum thickness, respectively, compared with the wild type (Fig. 5B and Table 2). This indicates that *cspV* affects biofilm formation more dramatically at 15°C but also shows a small but reproducible defect at 25°C.

In the Δ*cspV* mutant, we observed an upregulation of *rocS*, which encodes an important phosphodiesterase responsible for degrading c-di-GMP in *V. cholerae*; therefore, we hypothesized that *cspV* might regulate the expression of *vps* genes through modulation of c-di-GMP levels. Thus, cellular levels of c-di-GMP were quantified in the Δ*cspV* mutant before and 1 h after the shift from 37°C to 15°C and compared to those of the wild type. In agreement with previously published findings (22), we found that c-di-GMP levels were >1.5-fold higher ($P < 0.005$) at 15°C (postshift) than at 37°C (preshift) in wild-type *V. cholerae* (Fig. 5C). Yet, the Δ*cspV* mutant showed no significant increase in cellular levels of c-di-GMP at 15°C (postshift) compared to 37°C (preshift) ($P > 0.05$) (Fig. 5C). This suggests that *cspV* affects biofilm formation through the modulation of c-di-GMP, possibly through upregulation of the phosphodiesterase gene *rocS*.

**Regulation of the T6SS in the Δ*cspV* mutant.** To determine if CspV is involved in the regulation of the T6SS, we first compared *hcp* mRNA message abundance 1 h after a shift from 37°C to 15°C or 25°C using qRT-PCR of the Δ*cspV* mutant and wild type. We observed no difference (1.04-fold) in *hcp* mRNA message abundance between the Δ*cspV* mutant and the wild type at 15°C (Fig. 6A). However, at 25°C, there was a 14.0-fold decrease in *hcp* levels in the Δ*cspV* mutant compared to those in the wild type (Fig. 6B). Next, we analyzed Hcp production and secretion in the Δ*cspV* mutant grown at 25°C, the temperature at which we observed a difference in *hcp* mRNA abundance. We found that the Δ*cspV* mutant did not produce Hcp, similar to what occurred with the Δ*hcp* control strain (Fig. 6C). Finally, we compared levels of T6SS-mediated killing of *E. coli* between the Δ*cspV* mutant and wild-type *V. cholerae*. The Δ*cspV* strain exhibited a significant decrease in T6SS-mediated killing of *E. coli*, comparable to that of the Δ*hcp* mutant, at 37°C and 25°C, while no killing was observed at 15°C in any of the strains (Fig. 6D). These results show that *cspV* is required for T6SS-mediated killing under the conditions tested.

**Analysis of *D. magna* colonization by the Δ*cspV* mutant.** Because we determined that *cspV* is important for biofilm formation at temperatures that *V. cholerae* would experience in aquatic reservoirs, we hypothesized that *cspV* is required for the attachment of *V. cholerae* to zooplankton. To test this, we used *D. magna* as a model for zooplankton colonization, since *V. cholerae* has been shown to attach to the chitinous surface of *D. magna* (54), and *Daphnia* spp. have been found to coexist with *V. cholerae* in areas were cholera is endemic (55, 56). To determine if a strain lacking *cspV* has a colonization defect, *D. magna* was incubated with either wild-type *V. cholerae* or the *cspV* deletion mutant for 24 h and then imaged using scanning electron microscopy (SEM). We used *D. magna* incubated with a *V. cholerae* strain lacking the type IV pilus gene, *mshA*, as a control, since it was previously established that this mutant has an attachment defect on the surface of *D. magna* (54). We found single bacterial cells and aggregates of wild-type *V. cholerae* attached to the carapace of *D. magna*, primarily near the dorsal and ventral spinules (Fig. 7A). The Δ*cspV* mutant was found attached as single cells in the same regions, although at

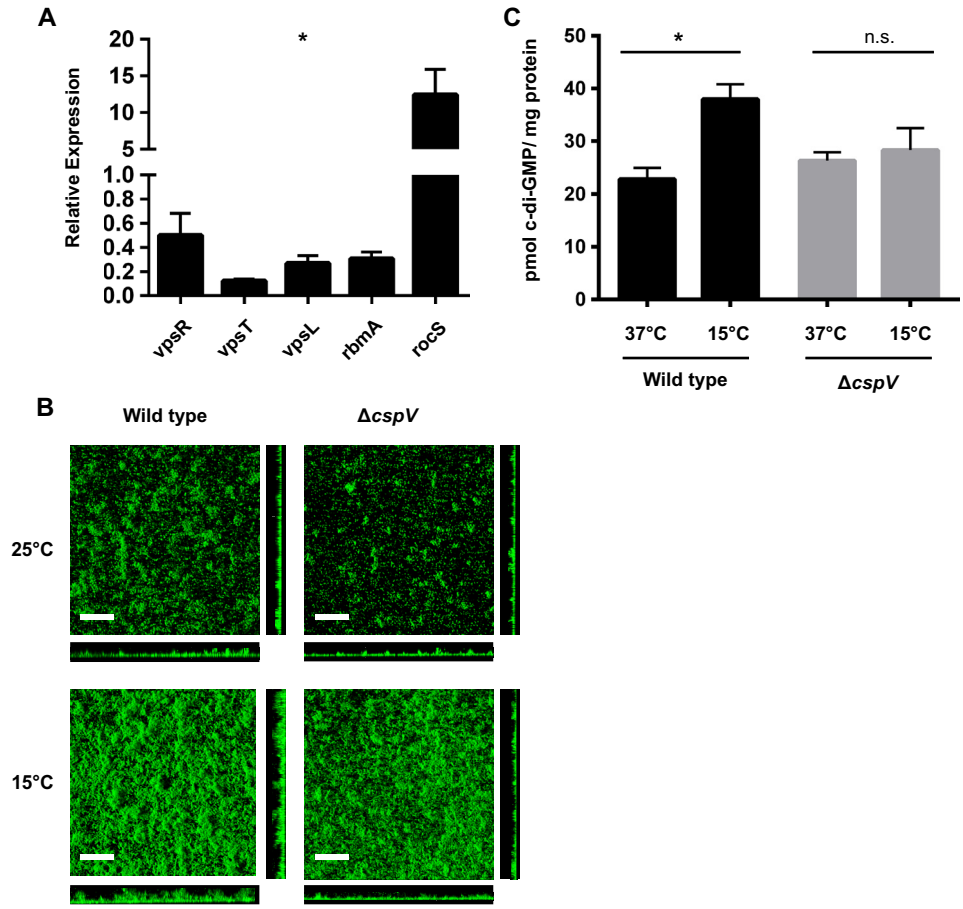


**FIG 5** *cspV* regulates biofilm formation. (A) Biofilm gene expression in the Δ*cspV* mutant compared with wild-type *V. cholerae* 1 h after a shift from 37°C to 15°C by qRT-PCR. All genes exhibited a statistically significant difference between the Δ*cspV* mutant and the wild type at 15°C (*,$P < 0.05$). Error bars indicate standard deviations of the results from four biological replicates. (B) Three-dimensional biofilm structures of wild-type *V. cholerae* and the strain lacking *cspV* formed at 25 and 15°C after 24 h of incubation. The images shown are from one representative experiment of three independent experiments. Scale bars represent 40 μm. (C) c-di-GMP levels in the Δ*cspV* mutant and wild-type *V. cholerae* grown at 37°C and then shifted to 15°C for 1 h. (A and C) Error bars indicate standard deviations of the results from four biological replicates. *, $P < 0.05$; n.s., not significantly different ($P > 0.05$).

drastically reduced numbers, and no aggregates were observed (Fig. 7A), similar to what was seen with an *mshA* mutant (see Fig. S1 in the supplemental material).

To further investigate the impact of *cspV* on plankton association, we analyzed the ability of both wild-type *V. cholerae* and the *cspV* deletion mutant to colonize zooplankton by individually infecting live *D. magna* cells with each strain and enumerating the surviving bacteria after 24 h. The mutant lacking *cspV* attached to *Daphnia* 9.8-fold less than the wild type (Fig. 7B), similar to the Δ*mshA* strain control, which attached 13-fold less than the wild type (Fig. 7B). In contrast, we did not see any difference in *V. cholerae* levels in planktonic cells, indicating that the Δ*cspV* mutant survives but does not attach to *Daphnia* (Fig. 7C). Collectively, these results show that *cspV* is important for attachment to chitinous surfaces of live zooplankton.

**TABLE 2** COMSTAT analysis of biofilms formed by WT and a strain lacking *cspV*

| Strain/ genotype | Temp (°C) | Biomass ($\mu m^3/\mu m^2$) | Thickness (μm) | | Roughness coefficient |
|---|---|---|---|---|---|
| | | | Avg | Maximum | |
| WT | 25 | 1.84 (0.76) | 1.78 (0.64) | 10.07 (3.22) | 1.05 (0.33) |
| | 15 | 4.61 (1.24) | 5.10 (1.55) | 15.64 (3.01) | 0.53 (0.10) |
| Δ*cspV* | 25 | 1.40 (0.92) | 1.17 (0.74) | 7.33 (2.69) | 1.16 (0.50) |
| | 15 | 2.26 (0.89) | 1.90 (0.53) | 5.67 (2.85) | 0.51 (0.35) |

[a] Biofilms were grown at 15°C and 25°C in static chambers in LB medium for 24 h. Values in parentheses are standard deviations.

**Type VI secretion-mediated killing on the surface of *D. magna*.** As shown above, the T6SS was highly active at 25°C (Fig. 3) and requires *cspV* (Fig. 6), suggesting that type VI-mediated competition may be important for surface colonization in aquatic environments. The chitinous surface of *D. magna* harbors a diverse microbial community (57). We hypothesized that *V. cholerae* would use its T6SS to kill the microbiota of *D. magna*. To address this, we used an *Aeromonas* sp. isolated from the *D. magna* microbiome as a prey strain to conduct T6SS *in vitro* killing assays. We observed a nearly 44-fold reduction in the number of CFU of *Aeromonas* sp. after incubation with wild-type *V. cholerae* at 25°C (Fig. 8A) and a much less dramatic reduction in the number of *Aeromonas* sp. CFU when this organism was incubated with the Δ*cspV* strain or the Δ*hcp* control strain (3.2- or 1.8-fold, respectively) (Fig. 8A). We then performed killing assays *in vivo* using

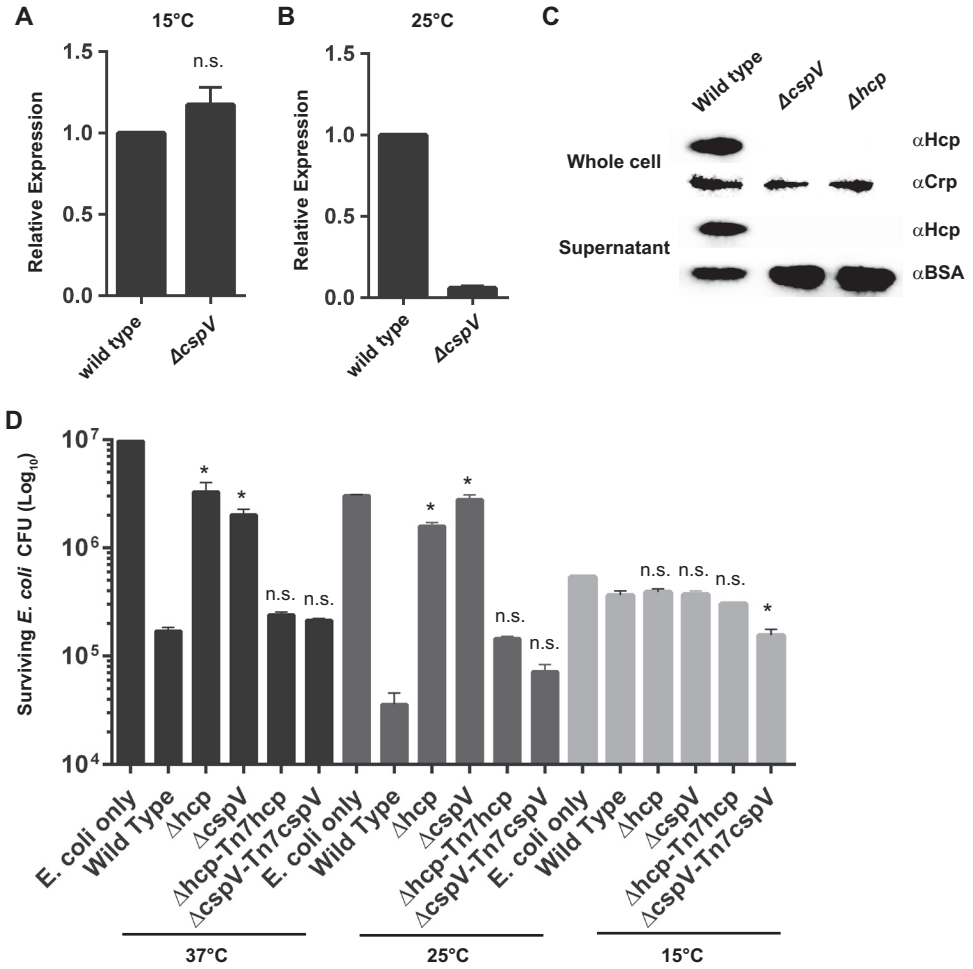


**FIG 6** CspV impacts the T6SS. Analysis of *hcp* expression by qRT-PCR in the Δ*cspV* mutant and wild-type *V. cholerae* upon a shift from 37°C to 15°C (A) or to 25°C (B). Error bars indicate standard deviations of the results from four biological replicates. (C) Hcp production levels by Western blot analysis in the Δ*cspV* mutant at 25°C. (D) T6SS-mediated killing of *E. coli* wild-type and mutant strains at 37°C, 25°C, and 15°C. Error bars indicate standard deviations of the results from three biological replicates. *, $P < 0.05$; n.s., not significantly different ($P > 0.05$).

live *D. magna* as the attachment surface. We found that there was a 23-fold decrease in the number of *Aeromonas* sp. cells when they were incubated with wild-type *V. cholerae* on the surfaces of live *D. magna* (Fig. 8B) and only a 1.2- or 1.4-fold change when they were incubated with the Δ*cspV* strain or the Δ*hcp* control strain, respectively (Fig. 8B). These results indicate that the *V. cholerae* T6SS can be used to kill zooplankton-associated bacteria and that *cspV* is crucial to this process.

## DISCUSSION

Very little is known about the mechanism by which *V. cholerae* senses and responds to varying temperatures or how this response impacts *V. cholerae* environmental survival. Like Datta and Bhadra (21), we observed a lag in growth after a low-temperature shift (see Fig. S2 in the supplemental material). We determined that *V. cholerae* exhibits significant alterations in its transcriptome in response to low-temperature shifts. Many of these changes in gene expression are presumably oriented toward overcoming the challenges imposed by cold shock. Studies aimed at characterizing the cold shock responses of other organisms have commonly identified a number of genes involved in DNA modulation, transport and binding functions, membrane fluidity, cell envelope modification, and energy metabolism (16, 58–63). We found similar genes that exhibited differential expression upon a low-temperature shift in *V. cholerae* as well (Table S1).

Little is known about the regulation and function of CSPs in *V. cholerae*. In *E. coli*, there are nine cold shock proteins; however, only four are cold inducible (16). Our study revealed that three of the four predicted cold shock genes, *cspV*, *cspA*, and, to a lesser extent, VC1142, were induced in *V. cholerae* upon a shift to low temperatures. Previous work on *cspV* in *V. cholerae* has indicated that it contains a cold-inducible promoter that lacks a long 5′-untranslated region in its mRNA transcript, an unusual exception among cold shock genes that are traditionally posttranscriptionally regulated (21). Transcriptional profiling of human-shed *V. cholerae* found *cspV* to be highly induced (64), further supporting the hypothesis that cold shock genes may play an important role in the infectious cycle of *V. cholerae* by promoting survival and persistence in the environment after dissemination from the human host. Other transcriptional profiling studies have found that *cspV* transcript levels are elevated in the presence of bile (65) and high

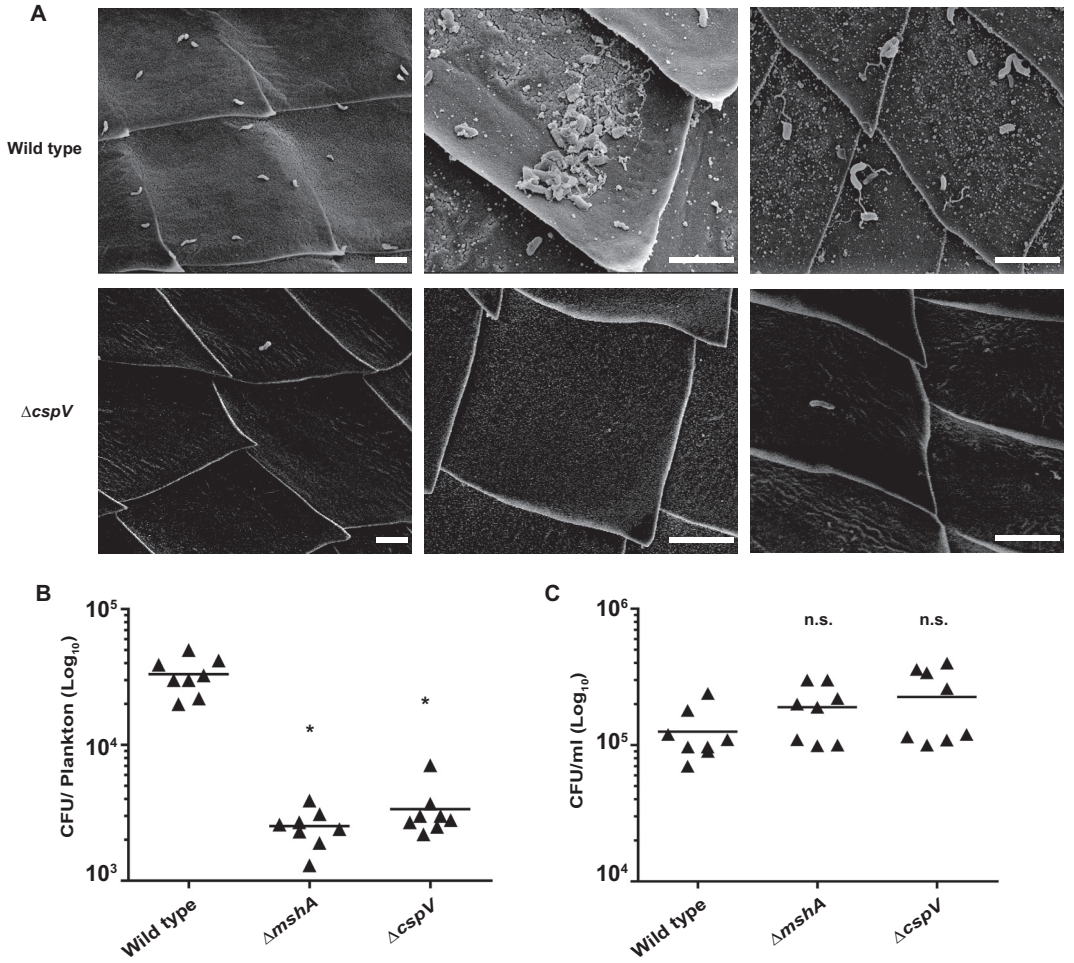


**FIG 7** CspV impacts attachment to live *D. magna*. (A) SEM images showing the wild type and Δ*cspV* mutant attached to *D. magna*. Scale bars represent 5 μM. (B and C) Analysis of the abilities of wild-type *V. cholerae*, the Δ*mshA* mutant, and the Δ*cspV* mutant to colonize *D. magna* (B) and survive in ADaM (C). Error bars indicate standard deviations of results from eight biological replicates. *, $P < 0.05$; n.s., $P > 0.05$.

c-di-GMP (35). We found that the deletion of this gene causes a significant defect in biofilm formation and T6SS in *V. cholerae* (Fig. 5 and 7). Further studies are needed to determine the molecular underpinnings of CspV-mediated regulation of mRNA abundance.

This study also revealed that the expression of biofilm genes and major regulators of biofilm formation is controlled by temperature fluctuations. Low-temperature growth has previously been determined to affect biofilm formation in multiple bacteria (66–69), and in *V. cholerae*, temperature affects biofilm formation though the modulation of c-di-GMP signaling by a set of six diguanylate cyclases in *V. cholerae* (22). Here, we have demonstrated that low-temperature biofilm gene expression is controlled by transcriptional modulation of known biofilm regulators of *aphA*, *hapR*, *vpsR*, and *vpsT* (Fig. 2A). Both VpsR and VpsT have been demonstrated to be receptors of c-di-GMP (70, 71); therefore, they may be the receptors responsible for relaying increased c-di-GMP signals to downstream processes at low temperatures; however, future studies will be needed to address this. We found that the expression of *aphA*, a major regulator of virulence (72), is significantly enhanced at a low temperature. To our knowledge, this is the first report of the modulation of this virulence regulator at 15°C, suggesting that AphA may play an important role outside the human host.

Our study also determined that temperature has a dramatic impact on the T6SS. We found that *hcp* message abundance, protein production and secretion, and activity were highest at 25°C (Fig. 3A and B). It was previously reported that Hcp is increased in cells grown at 23°C compared to levels at 37°C (48), further corroborating our results. A molecular mechanism by which *hcp* transcription is decreased at 15°C is yet to be determined. However, we surmise that decreased levels of *hapR* at lower temperatures (Fig. 2A) might in part be responsible, as HapR positively regulates *hcp* expression (45, 73).

Water temperature and abundance of zooplankton covary with *V. cholerae* prevalence in the aquatic environment (5, 10, 11). Attachment to zooplankton is advantageous to *V. cholerae* in a multitude of ways. First, attached *V. cholerae* cells are able to utilize chitin as a source of carbon and nitrogen (74, 75). Second, contact with chitin induces type IV pili required for competence (76, 77), allowing *V. cholerae* to potentially increase its fitness by taking up and incorporating foreign DNA when it is closely associated with other bacteria on the zooplankton surface. Finally, attachment to zooplankton allows *V. cholerae* to disseminate,

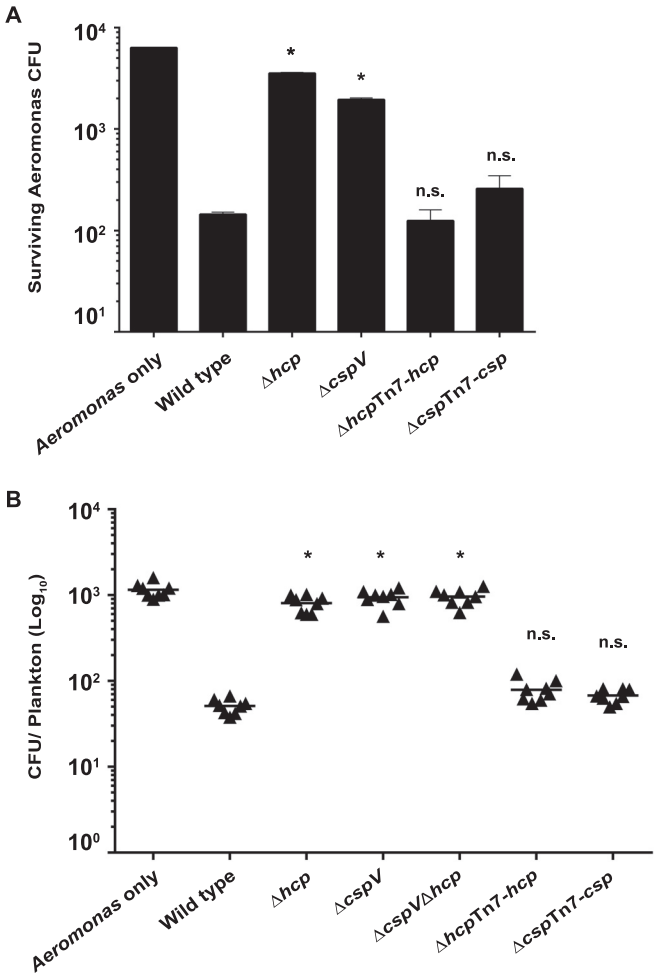


**FIG 8** CspV contributes to interspecies killing on the surface of live *D. magna*. T6SS-mediated killing of *Aeromonas* sp. *in vitro* (A) and on the surface of *D. magna* (B). Error bars indicate standard deviations of the results from eight biological replicates. *, $P < 0.05$; n.s., not significantly different ($P > 0.05$).

thereby increasing the chance of exposure to human hosts, and it has been shown to be important in transmission (23, 24, 26, 78). We found that CspV is important for the attachment to live zooplankton through direct microscopic observation and numbers of CFU recovered from colonization experiments (Fig. 7). In addition, we determined that CspV is required for type VI-mediated killing of an *Aeromonas* sp., the microbiome member isolated from *D. magna*, and has been demonstrated to affect the growth, survival, and reproduction of *D. magna* (57). This also may indicate that the presence of *V. cholerae* has implications on the health of zooplankton through alterations in microbiome composition.

*V. cholerae* is a human pathogen with an aquatic life cycle. It therefore offers a great opportunity to study the effects of environmental variables, such as temperature, on the environmental survival and transmission of an infectious agent. Our studies provide new insights into the adaptation of *V. cholerae* to fluctuating environmental parameters and reveal how this adaptation impacts the environmental survival of this important human pathogen.

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We declare no conflicts of interest.

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