environmental microbiology

Environmental Microbiology (2020) **22**(10), 4409–4423 doi:10.1111/1462-2920.15141

Elevated levels of VCA0117 (VasH) in response to external signals activate the type VI secretion system of *Vibrio cholerae* O1 El Tor A1552

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Summary

The type VI nanomachine is critical for *Vibrio cholerae* to establish infections and to thrive in niches co-occupied by competing bacteria. The genes for the type VI structural proteins are encoded in one large and two small auxiliary gene clusters. VCA0117 (VasH) – a σ^{54}-transcriptional activator – is strictly required for functionality of the type VI secretion system since it controls production of the structural protein Hcp. While some strains constitutively produce a functional system, others do not and require specific growth conditions of low temperature and high osmolarity for expression of the type VI machinery. Here, we trace integration of these regulatory signals to the promoter activity of the large gene cluster in which many components of the machinery and VCA0117 itself are encoded. Using *in vivo* and *in vitro* assays and variants of VCA0117, we show that activation of the σ^{54}-promoters of the auxiliary gene clusters by elevated VCA0117 levels are all that is required to overcome the need for specialized growth conditions. We propose a model in which signal integration via the large operon promoter directs otherwise restrictive levels of VCA0117 that ultimately dictates a sufficient supply of Hcp for completion of a functional type VI secretion system.

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Introduction

Vibrio cholerae species are diverse with respect to the virulence determinants they encode. Those of pandemic strains, serogroups O1 and O139, carry *ctxAB* cholera toxin genes and the *tcp* genes for toxin-regulated pili – two major virulence determinants of the diarrheal disease cholera. Non-O1, non-O139 *V. cholerae* (NOVC strains), on the other hand, comprise a large group of environmental isolates that typically lack the *ctx* and *tcp* genes and cause gastroenteritis by less well-defined mechanisms (Kaper *et al*., 1995). More recently, the type VI secretion system (T6SS) – a contractile multicomponent nanomachine – has emerged as a potent environmental fitness factor of *V. cholerae* and many other Gram-negative pathogens (Pukatzki *et al*., 2006).

Vibrio cholerae uses its T6SS syringe-like apparatus to translocate proteins into the external milieu (e.g. Wang *et al*., 2015; Si *et al*., 2017), and directly inject effector proteins into both eukaryotic and bacterial cells to subvert their functioning (reviewed in the study by Joshi *et al*., 2017; Lien and Lai, 2017). Many components of the T6SS contractile puncturing device are structural and functional counterparts of the DNA injection machinery of bacteriophage tails (Basler, 2015; Bock et al., 2017 and references therein). The inner tube of the T6SS apparatus is composed of a rigid polymer of the haemolysin coregulated protein (Hcp), which is encapsulated by an outer contractile sheath formed by polymers of VipA and VipB. *Trimeric* VgrG proteins (valine-glycine repeat protein G) lie at the cell-puncturing tip and are sharpened by PAAR (proline-alanine–alanine-arginine) repeat proteins that are critical in aiding effector protein delivery (Burkinshaw *et al*., 2018).

Sheath contraction drives the syringe-like tube and tip into target cells to deliver multiple effector proteins by a number of different means. Structural proteins, such as Hcp, VgrG and PAAR, have amino- or carboxy-terminal effector domains, while other effectors bind VgrG and Hcp as a mode of delivery. The targets and mechanisms of action of different effector proteins are likewise diverse, being anti-bacterial, anti-eukaryotic or both (Russell *et al*., 2014). This versatility underlies the key roles of the

V. cholerae T6SS not only in pathogenicity but also in evading predation by eukaryotes, e.g. *amoeba*, *nematodes* and grazing by flagellates and ciliates (Pukatzki et al., 2006; Vaitkevicius et al., 2006), and for both inter- and intra-bacterial competition for niche domination in polymicrobial environments (e.g. Unterweger et al., 2012; Logan et al., 2018).

T6SS-dependent bacterial competition is believed to be a major driver of *genotypic* diversity in *V. cholerae* (Kostiuk *et al*., 2017). In all *V. cholerae* strains, structural components of the T6SS apparatus are encoded in one large and two small gene clusters. However, regulation of their expression varies between strains and is interwoven with the global regulatory networks of quorum sensing (via HapR, LuxO and TsrA) and the cAMP-CRP regulon (e.g. Ishikawa *et al*., 2009; Zheng *et al*., 2010; Shao and Bassler, 2014). Irrespective of global regulatory input, one specific and obligatory regulator for T6SS expression has been identified, namely VCA0117, also known as VasH.

VCA0117 belongs to the specialized family of bacterial enhancer binding proteins (bEBPs) and is required for production of Hcp encoded within the two small gene clusters (Bernard *et al*., 2011; Kitaoka *et al*., 2011). Members of the bEBP-family are mechano-transcriptional activators that utilize ATP hydrolysis to activate transcription mediated by the alternative σ-factor σ^{54} (also known as RpoN). Typically, the activities of bEBPs are themselves controlled in response to environmental cues through their N-terminal domains (Shingler, 2011; Bush and Dixon, 2012). However, despite its critical importance for expression of Hcp, without which the T6SS does not function (Ishikawa *et al*., 2012), little is known about how, or if, the activity of VCA0117 is controlled or how it targets DNA to regulate transcription.

Studies of *V*. *cholerae* T6SS-dependent functions have predominantly used the *V*. *cholerae* NOVC (O37 serogroup) V52 strain because it expresses the apparatus constitutively and can kill a wide range of Gram-negative bacteria (Pukatzki *et al*., 2006; MacIntyre *et al*., 2010). Unlike NOVC strains, most of the cholera-causing O1 strains were originally thought to have non-functional *T6SSs*; however, it was subsequently shown that O1 strains require environmentally relevant signals of high-osmolarity (340 mM NaCl) or low temperature (23°C) in order for Hcp, and therefore a functional *T6SS* machinery, to be expressed (Ishikawa *et al*., 2012).

In this work, we aimed to decipher at what level the signals of high osmolarity and low temperature are integrated to control production of a functional *T6SS* and the role of VCA0117 in this process. We present evidence for the binding sites of VCA0117 within the P*hcp* promoters that drive expression of *hcp* and a positive role for the DNA bending protein integration host factor (IHF) on VCA0117-dependent transcription from these promoters. Importantly, we show that the signals of high osmolarity and low temperature elevate transcription of the large gene cluster within which VCA0117 is encoded and further, that elevated levels of VCA0117 are in themselves sufficient to overcome the requirement for conditional growth signals to produce a functional *T6SS* apparatus.

Results

An active A-domain truncate of VCA0117 mediates signal-independent expression of Hcp

VCA0117 (VasH) is encoded within the large *T6SS* gene cluster (*vca0107* to *vca0123*) of chromosome II in *V. cholerae* El Tor strain A1552 (Fig. 1A). This σ^{54}-dependent transcriptional activator has been implicated in controlling RpoN (σ^{54})-dependent transcription of two bicistronic operons (*hcp-I-vgrG-1* of chromosome I and *hcp-2-vgrG-2* of chromosome II) that encode identical Hcp proteins and two of the three VgrG proteins in *V. cholerae* (Ishikawa *et al*., 2009; Bernard *et al*., 2011; Kitaoka *et al*., 2011; Dong and Mekalanos, 2012). To verify this regulatory circuitry in *V. cholerae* A1552, we generated a VCA0117 null derivative in which the majority of *vca0117* was deleted and analysed Hcp levels in whole cells and supernatants. Because the *T6SS* in A1552 is induced by low temperature and high salt conditions (Ishikawa *et al*., 2012), cells were cultured under non-inducing (LB at 37°C with 85 mM NaCl) and fully inducing conditions for T6SS (LB at 23°C with 340 mM NaCl). As anticipated, lack of VCA0117 resulted in undetectable levels of Hcp even after induction of gene expression, which allows both production and efficient secretion of Hcp by the wild-type strain (Fig. 1B, left).

VCA0117 has a signature short coiled-coil motif in the B-linker regions between the N-terminal A-domain and the AAA^+ C-domain involved in activating σ^{54}-RNA polymerase (Figs. 1A and S1). Where known, possession of a coiled-coiled like B-linker is indicative of a de-repression mode of control whereby deletion of the N-terminal domain results in constitutively active bEBPs (O'Neill *et al*., 2001; reviewed in the study by Shingler, 2011). Therefore, to verify that the phenotype of the VCA0117 null strain was not attributable to indirect effects on upstream or downstream genes within the *vca0107* to *vca0123* operon, we complemented this strain by introducing a plasmid expressing an N-terminal A-domain truncate (ΔA-VCA0117-His that lacks residues 1–76) from the P*tet* promoter. As shown in Fig. 1B (right), expression of ΔA-VCA0117-His resulted in hyper-production and secretion of Hcp in both the wild-type and the VCA0117 null strains even under non-inducing growth conditions.

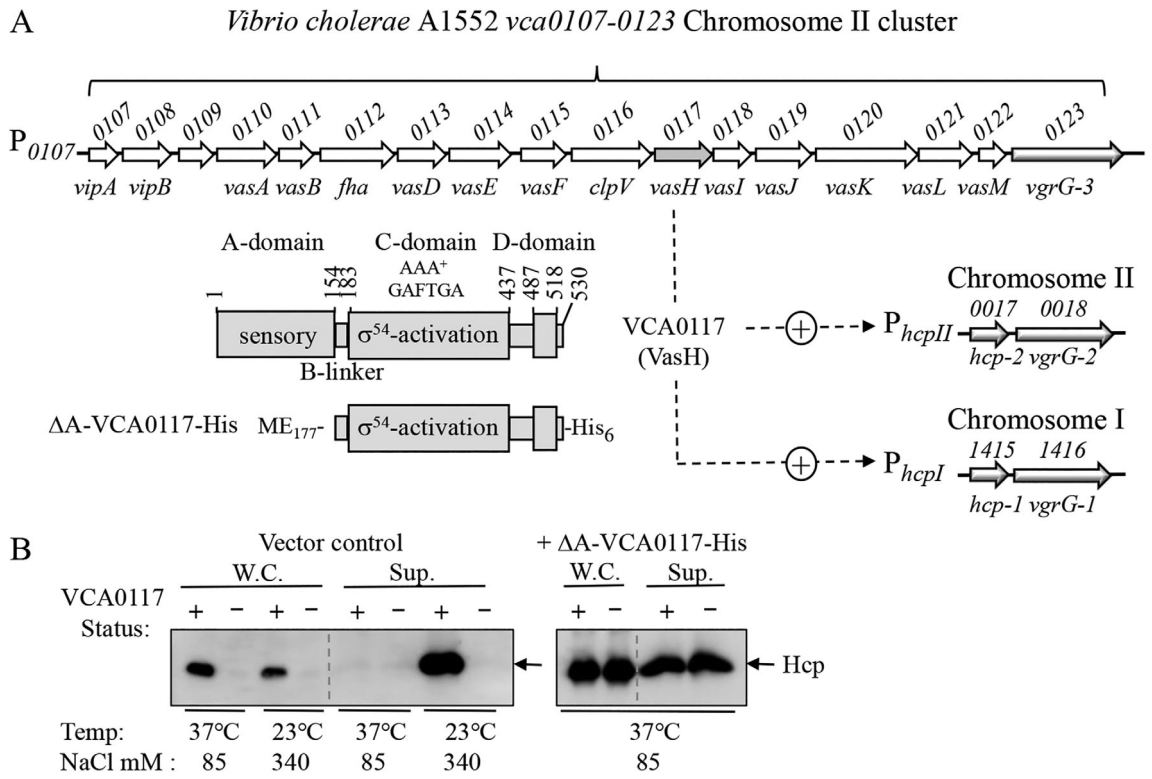

Fig 1. Genomic organisation and the Hcp regulatory circuit of VCA0117 (VasH).
A. Upper: genomic organisation of the large T6SS chromosome II cluster of *V. cholera* A1552 with the *vca0117* (*vasH*) gene highlighted in grey. Lower left, the domain structure of VCA0117 and a constitutively active variant (ΔA-VCA0117-His). As detailed in the Supporting Information Figs. S1 and S8, ΔA-VCA0117-His completely lacks it is a domain and has a non-native Met fused to the native Glu_{177} of VCA0117. Lower right, schematic of the VCA0117-mediated regulatory circuit with positive transcriptional control of the P*hcp* promoters that drive transcription of two bi-cistronic operons indicated by a +. Genes that encode identical Hcp proteins and variant VgrG proteins are highlighted by grey shadowing.
B. Immuno-detection of the 23 kDa Hcp protein in whole cell lysates (W.C.) and supernatants (Sup.) of *V. cholerae* strains harbouring either a vector control (pVI2403, left hand panel) or the P*tet* driven ΔA-VCA0117-His expression plasmid (pVI2431, right hand panel). Strains, wild-type *V. cholerae* A1552 (+) or its isogenic VCA0117 null derivative (–), were cultured in LB under non-inducing (37°C, 85 mM NaCl), or fully inducing (23°C, 340 mM NaCl) conditions as indicated.

Taken together, these results (i) recapitulate the low temperature and high-osmolarity induction requirements for *V. cholerae* A1552 T6SS expression of the wild-type strain; (ii) demonstrate the obligate dependency on VCA0117 for Hcp production and secretion; and importantly, (iii) show that ectopic expression of an active A-domain truncate of VCA0117 is sufficient to overcome signal-responsive control of T6SS in this strain. The latter finding provided the first suggestive evidence that VCA0117 activity and/or levels are a key factor in the control of expression of a functional T6SS in *V. cholerae* A1552.

Direct VCA0117-mediated regulation of Hcp is dependent on RpoN (σ^{54}) and IHF

Having established that VCA0117 is obligatory for expression of Hcp, we next turned our attention to the promoter regions controlled by this protein. The promoters of the two *hcp-vgrG* operons (P*hcpI* and P*hcpII*) are very similar, differing in only two positions in their common region from −130 to +1 relative to their predicted transcriptional start sites (Fig. 2A). In *V. cholerae* V52 – which constitutively expresses the T6SS apparatus – *in silico* analysis and σ^{54}-RNA polymerase binding experiments also implicated VCA0117 in σ^{54}-dependent control of the large T6SS operon driven by the P_{0107} promoter (Bernard *et al*., 2011). However, subsequent analysis of an RpoN (σ^{54}) null derivative of V52 found no alteration in transcription from this promoter (Dong and Mekalanos, 2012). Therefore, to clarify this issue for *V. cholerae* A1552, we developed a dual plasmid transcriptional reporter system consisting of one plasmid expressing a VCA0117 derivative and the other carrying the bacterial luciferase reporter genes (*luxAB*) under the control of a test promoter. Promoter output was then screened using a simple luciferase (light emittance) plate test assay as detailed in Experimental Procedures.

Expression of ΔA-VCA0117-His elicited readily detectable light emission from the P*hcpII-luxAB* promoter reporter in *Escherichia coli* (strains 3 and 4, Fig. 2B). In

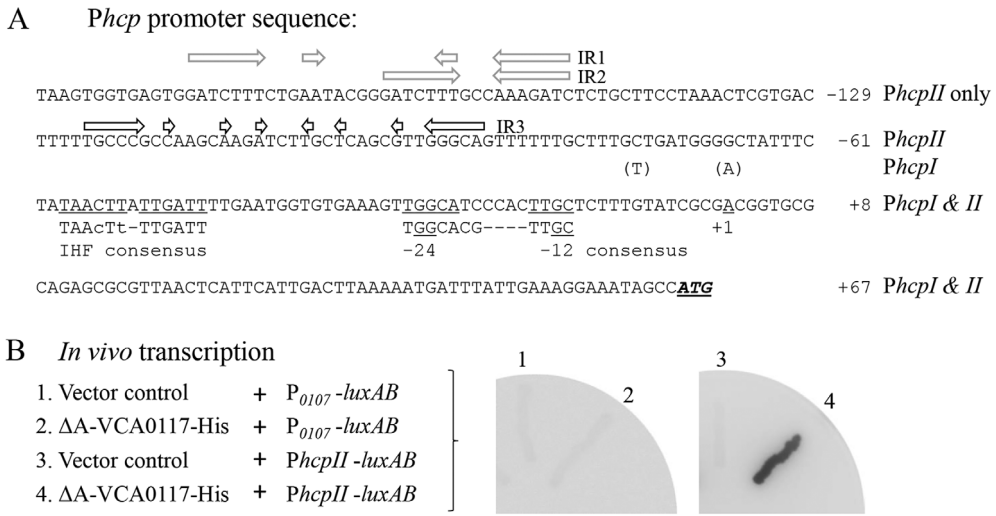

Fig 2. VCA0117 controls transcription from P*hcpII*, but not the P$_{0107}$ promoter.
A. Sequences of the P*hcp* promoter regions of chromosome I and II with the ATG initiation codon shown in bold italics. The highly conserved −128 to +67 regions only differ at two positions (bases shown in parenthesis). Co-ordinates are given relative to the predicted +1 transcriptional start from the −24, −12 promoter that is shown aligned with the consensus σ^{54} binding motifs (Barrios *et al*., 1999). A potential target site for IHF is located upstream of the promoters and is shown aligned with the reverse complement of the core 13 bp of the IHF-binding consensus sequence (AATCAA-aAgTTA; Goodrich *et al*., 1990). Lowercase letters indicate less conserved bases and hyphens indicate any base. Light grey open arrows indicate the extent of two inverted repeats that are only found in the P*hcpII* upstream region (IR1 and IR2), while the dark grey open arrows indicate IR3 that is common to both promoters.
B. Luciferase plate tests of VCA0117-mediated *in vivo* transcription with a dual plasmid system in *E. coli* SoluBL21. Strains contained either vector control (pVI2403) or the P*tet* expression plasmid for ΔA-VCA0117-His (pVI2431) and either the luciferase transcriptional reporter for the large gene cluster (P$_{0107}$-*luxAB*; pVI2448) or the P*hcpII-luxAB* transcriptional reporter (pVI2441) as indicated on the left. Strains were streaked onto LA plates (85 mM NaCl), supplemented with carbenicillin and chloramphenicol and incubated at 37°C.

contrast, no transcription above that of a vector control was detectable with the P$_{0107}$-*luxAB* reporter (strains 1 and 2, Fig. 2B). We infer from these data that VCA0117 directly drives transcription from the P*hcp* promoters, but does not influence transcription from the P$_{0107}$ promoter of the large gene cluster that encodes VCA0117, which agrees with data derived from an RpoN null derivative of *V. cholerae* V52 (Dong and Mekalanos, 2012).

The P*hcp* promoters contain classic σ^{54}-dependent −24, −12 promoter motifs and also a putative binding site for the DNA bending protein integration host factor (IHF, Fig. 2A), which in other systems aids physical interaction between bEBPs and promoter-bound σ^{54}-RNA polymerase. We used the same dual plasmid transcriptional reporter system as described above in both *E. coli* and *V. cholerae* A1552 to verify that VCA0117-mediated transcriptional activation from the P*hcpII* promoter is dependent on these two proteins. As shown in Figs. 3A and B, lack of RpoN (σ^{54}) abolished any detectable transcription from P*hcpII* in both organisms, demonstrating that the P*hcpII* -196 to +26 region does not carry any additional intrinsically active promoters dependent on other σ-factors. In the case of IHF, lack of this DNA-bending protein severely compromised transcription, reducing P*hcpII* output to <1% and ~10% of that observed in wild-type *E. coli* and *V. cholerae* respectively. Hence, both proteins are required for efficient VCA0117-dependent output from P*hcpII*.

Consistent with the findings above, analysis of wild-type *V. cholerae* A1552 and its isogenic IHF-null and RpoN (σ^{54}) derivatives showed that lack of either σ^{54} or IHF lead to undetectable levels of Hcp (Fig. 3C). Expression of Hcp has previously been found to be growth phase regulated, being maximal at an OD$_{600nm}$ of 2.0–3.0, after which it declines to undetectable levels (Ishikawa *et al*., 2009, and see Supporting Information Fig. S3). Given the absolute dependency of Hcp expression on σ^{54}, we also analysed expression levels of σ^{54} at different phases of growth. As shown in Fig. S3, σ^{54} levels follow a similar expression pattern as those of Hcp, suggesting that available σ^{54} for formation of the σ^{54}-RNA polymerase holoenzyme plays a role in locking maximal expression of Hcp to the exponential-to-stationary phase of growth.

VCA0117-mediated regulation of Hcp requires an upstream DNA inverted repeat

The requirement for IHF for efficient transcription from P*hcpII* suggests that, akin to other bEBPs, VCA0117 acts via binding to distally located upstream activation

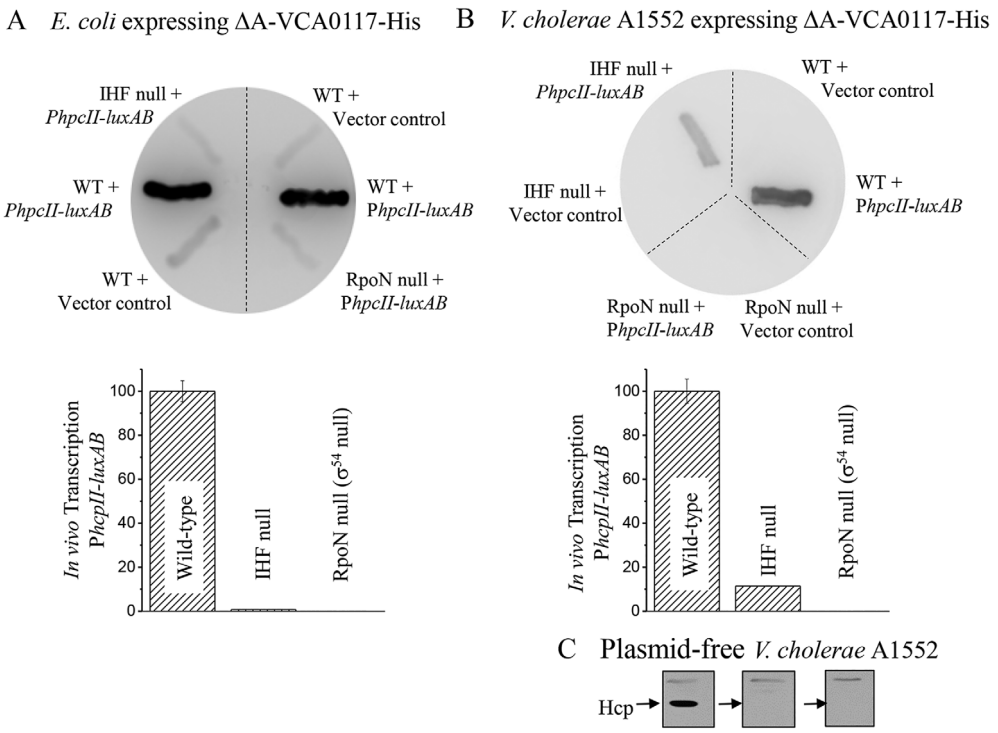

Fig 3. VCA0117-mediated regulation is dependent on RpoN (σ^{54}) and IHF.
A. Upper, luciferase plate tests of VCA0117-mediated *in vivo* transcription with a dual plasmid system in *E. coli* (strains: MG1655 and its RpoN null counterpart; S90C and its IHF null derivative). All strains contained the P*tet* ΔA-VCA0117-His expression plasmid (pVI2431) and either the P*hcpII-luxAB* luciferase transcriptional reporter (pVI2441) or its cognate vector control (pVI928). Strains were streaked onto LA plates supplemented with carbenicillin and chloramphenicol and incubated at 37°C. Lower, graphed values correspond to luciferase activities of strains cultured in liquid LB (85 mM NaCl) at 30°C for 6 h to early stationary phase (OD_{600nm} of ~3.0). Values are the average of triplicate determinations from two independent experiments ± SE, obtained after subtraction of background levels in the corresponding vector control strain. Experiments were normalised by setting the values for the wild type as 100. Absolute values of the wild type: 6.3×10^{-6} RLU/A_{600} for MG1655; 6.2×10^{-6} RLU/A_{600} for S90C. *P*-values, calculated using Welch's two-tailed *t*-test between wild type and null strains, are ≤1e-08. Complementation of the IHF null and RpoN strains to restore transcription from P*hcpII* is shown in the Supporting Information Fig. S2.
B. As under panel A, but with *V. cholerae* A1552 and its isogenic RpoN and IHF null derivatives. Absolute values for wild-type *V. cholerae* A1552: 1.3×10^{-5} RLU/A_{600}. *P*-values, calculated using Welch's two-tailed *t*-test between wild type and null strains, are ≤1e-05.
C. Immuno-detection of Hcp whole-cell preparations of plasmid free wild-type *V. cholerae* A1552 and its RpoN and IHF null derivatives. The cropped images are derived from samples analysed and processed on the same gel.

sequences (UASs). These UASs are usually composed of imperfect inverted repeats located 100–200 bp upstream of the cognate promoter they control (Cases *et al*., 2003; Bush and Dixon, 2012). Examination of the DNA sequences of *PhcpI* and P*hcpII* revealed three long imperfect inverted repeats, two specific to P*hcpII* (IR1 and IR2, Fig. 2A), and one common to both promoters (IR3, Fig. 2A). Given that detectable Hcp expression is abolished in the absence of VCA0117, this suggested that the common IR3 repeat would serve as the DNA binding sites from which VCA0117 acts to promote transcription by σ^{54}-RNA polymerase. To directly test this idea, we generated a series of transcriptional reporters that progressively lack larger portions of the promoter upstream region as depicted in Fig. 4A and assayed their performance in *E. coli* expressing ΔA-VCA0117-His.

The first deletion derivative, which lacks IR1 and IR2, reflects both P*hcpI* and P*hcpII* (see Fig. 2A). This derivative showed output comparable to the reporter carrying DNA encompassing all three IRs (compare 1 with 2 and 3, Fig. 4B). Deletion of about half of IR3 decreased output to ~30% of that seen with the full IR3, while deletion of DNA encompassing all of IR3 resulted in background transcription equivalent to the vector control (compare 1 with 4 and 5, Fig. 4B). Residual transcription observed with the reporter carrying DNA spanning the promoter-proximal half of IR3 was further reduced by mutations that retained the same nucleotide composition, but shuffled the repeat motif (compare 4 and 6, Fig. 4B). Based on these data, we designated the two halves of IR3 as UAS1 and UAS2, respectively.

To further analyse the requirements for VCA0117-mediated transcription of the P*hcp* promoters, we performed single round *in vitro* transcription assays with purified ΔA-VCA0117-His and supercoiled DNA template bearing P*hcpII* with different extents of upstream

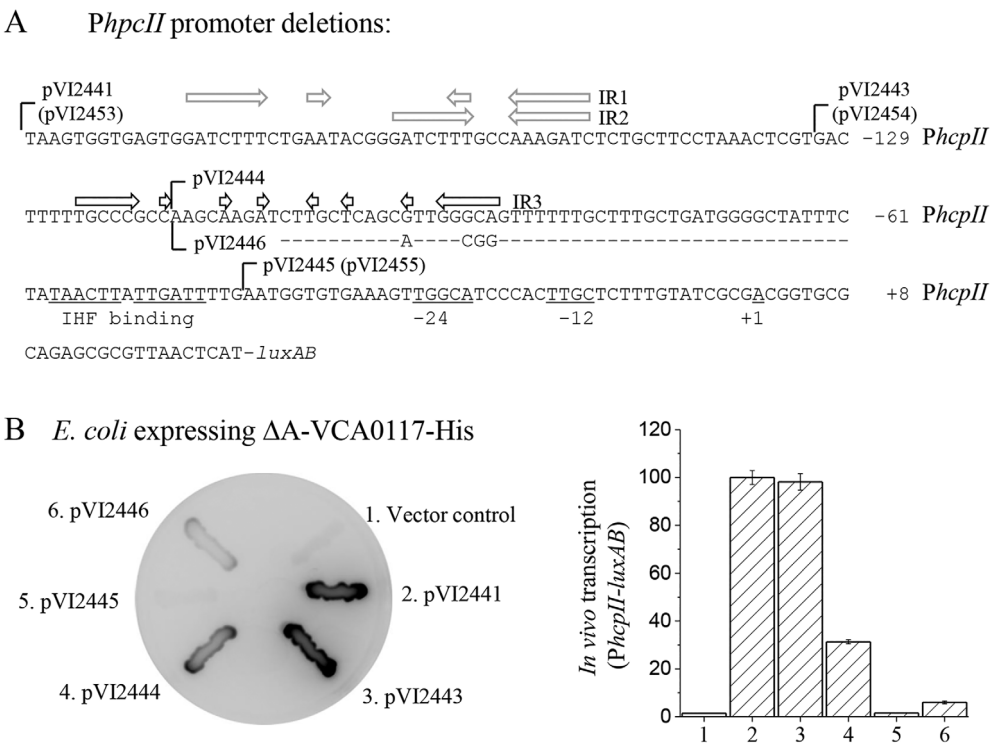

Fig 4. Inverted repeat 3 (IR3) is required for VCA0117 -mediated regulation.
A. Schematic illustration of the extent of the 5′-upstream sequences in P*hcpII-luxAB* transcriptional reporters used in panel B. Plasmid names in brackets are for the corresponding promoter inserts carried by the *in vitro* transcription templates used in Fig. 5.
B. Plate (left) and quantitative (right) luciferase assays of *E. coli* DH5 harbouring the ΔA-VCA0117-His expression plasmid (pVI2431) and either the indicated P*hcpII-luxAB* luciferase reporter or the cognate vector control (pVI928). Assays were performed as described under Fig. 3A. Graphed values are of exponential phase cultures (OD_{600nm} = 0.3 ± 0.15) and represent the average ± SE of five determinations from each of two independent cultures, normalized by setting the value of pVI2441 as 100. *P*-values of strain 3 compared to strains 1, 4, 5, or 6, were all ≤1e-07 as determined by the Tukey–Kramer's test.

DNA (Fig. 5A). In the presence of 20 nM *E. coli* σ^{54}-RNA polymerase and IHF, near saturating output from the full-length P*hcpII* promoter was achieved with 300 nM of ΔA-VCA0117-His (Fig. 5B). We next compared the ability of ΔA-VCA0117-His to promote transcription using DNA templates bearing the full length P*hcpII* promoter with those carrying either UAS1 and 2 (i.e. IR3, present in both *hcp* promoters) or just the −24, −12 σ^{54}-promoter alone. With 100 nM ΔA-VCA0117-His, transcription was completely dependent on the presence of the UASs of IR3 (Fig. 5C), as was observed *in vivo* (Fig. 4B, compare 2, 3 and 5).

Expression of ΔA-VCA0117-His mediates signal-independent inter-bacterial killing

Signal-responsive control of T6SS expression in A1552 was originally detected through elevated Hcp levels in response to low temperature and high osmolarity growth conditions (as in Fig. 1B). In addition, qRT-PCR analyses of transcripts of representative T6SS genes revealed ∼1.2- to 2-fold elevated levels of transcripts of the large T6SS operon genes driven by the P_{0107} promoter, as well as elevated levels of transcripts of the two *hcp-vgrG* operons, in response to high osmolarity (Ishikawa *et al*., 2012). Because expression of ΔA-VCA0117-His overcame the need for these conditional growth signals to efficiently produce and secrete Hcp (Fig. 1B), we reasoned that these cues were likely orchestrated through the activity of the P_{0107} promoter to produce increased levels of components of the T6SS apparatus and VCA0117. Elevated levels of VCA0117 might then in turn be sufficient to activate transcription from the P*hcp* promoters to provide sufficient Hcp and VgrG proteins for final assembly of a functional T6SS apparatus.

To test the above idea, we introduced the luciferase P_{0107} promoter reporter plasmid into *V. cholera* A1552 and monitored promoter activity on rich media plates containing either 85 or 340 mM NaCl and grown under normal (37°C) or low (23°C) temperature conditions. As can be seen in Fig. 6A, both high osmolarity (340 mM NaCl) and growth at low temperature (23°C) independently elicited elevated transcription from P_{0107}. Intriguingly, quantitative luciferase assays performed under the same conditions – but in liquid media – did not detect notable changes in P_{0107} activity (Supporting Information Fig. S4).

As expanded upon in the Discussion section, this latter finding suggests that one or more of the regulatory elements that control this response from the P_{0107} promoter requires surface contact as a regulatory signal.

Taken together, the data in Figs. 1 and 6A suggest that the conditional growth signals of low temperature and high osmolarity are integrated through the activity of the P_{0107} promoter to allow VCA0117-dependent production of a functional T6SS apparatus. If this were the case, simple ectopic expression of ΔA-VCA0117-His would be anticipated to bypass the need for either of these regulatory signals for T6SS functionality. To test this prediction, we performed an inter-bacterial killing assays in which a fully functional T6SS machinery is required to deliver effector proteins from *V. cholerae* A1552 (predator) into *E. coli* (prey). Within these assays, each species is grown to an OD_{600nm} of 2.0 to 3.0 and equal numbers of each then mixed and co-incubated on solid media for four hours under different osmolarity and temperature conditions. Subsequent determinations of the 'killing index' reflect the T6SS-dependent killing proficiency of *V. cholerae*.

As shown in Fig. 6B (left), the efficiency of *V. cholerae* A1552 in killing *E. coli* recapitulates signal-dependent transcription from the P_{0107} promoter, with both low temperature (23°C) and high osmolarity (340 mM NaCl) having independent and additive effects. Notably, under both growth temperature conditions, ectopic expression of ΔA-VCA0117-His increases killing proficiency to a level similar to that observed under high osmolarity conditions, i.e. only up to or just exceeding the maximum that the wild-type could achieve at each growth temperature (Fig. 6B, right). These data provides strong support for the notions (i) that conditional growth signals are integrated through the activity of the P_{0107} promoter to relieve otherwise limiting levels of VCA0117, and (ii) that subsequent VCA0117-controlled expression of the two *hcp-vgrG* operons is the determinant that dictates if sufficient functional T6SS apparatus competent to mediate efficient inter-bacterial killing can be produced.

Full-length VCA0117 variants are active in vivo *and* in vitro

The data shown in Figs. 1–6 all employed the transcriptional promoting ability of the A-domain truncate ΔA-VCA0117-His. As alluded to in the proceeding sections, the N-terminal A-domains of bEBPs, where present, usually exert control on the activity of the cognate bEBP. *In silico* searches of domain and motif databases using the 154 amino acid sequences of the A-domain of VCA0117 (Supporting Information Fig. S1) did not identify any signature motifs. However, a BLASTP search with an E value cut-off of 0.01 identified 239 homologues in genomes of aquatic bacteria belonging to three orders of γ-proteobacteria, the *Vibrionales*, *Oceanospirillales* and *Alteromonadales*, with sequence identity from 25% to 99%. As detailed in Experimental Procedures, multiple sequence alignment of these protein enabled more sensitive sequence searching, which suggests that this region of VCA0117 is a GAF-domain – named after cGMP-specific phosphodiesterases, adenylyl cyclases and the bEBP FhlA.

Comparison of $VCA0117_{A1552}$ with its counterpart from *V. cholerae* V52, which constitutively expresses the T6SS apparatus, identified two variant residues. One of

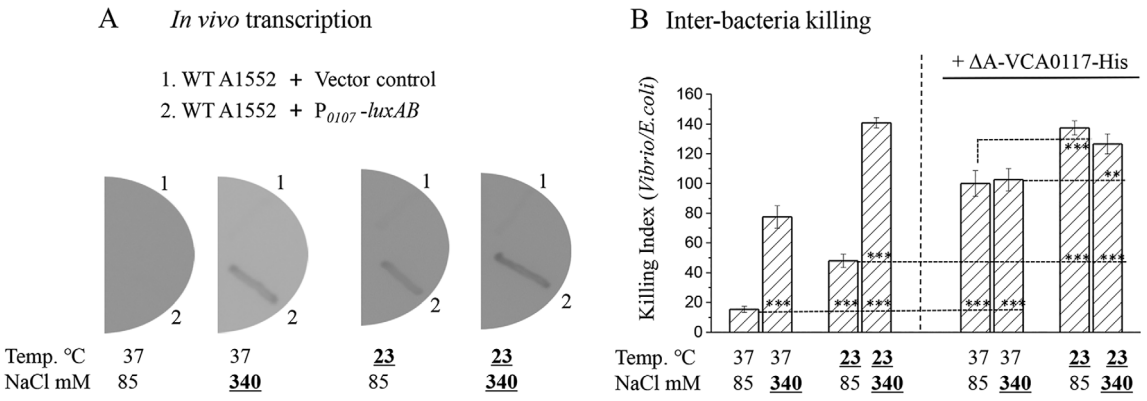

Fig 6. Signal-responsive T6SS-dependent killing is overcome by constitutively active ΔA-VCA0117-His.
A. Luciferase plate assays of *V. cholerae* A1552 harbouring either the transcriptional reporter for the large gene cluster (P_{0107}-*luxAB*; pVI2448) or its cognate vector control (pVI928). Strains were streaked onto LA plates containing 85 or 340 mM NaCl, supplemented with carbenicillin and incubated at either 37°C or 23°C.
B. T6SS-dependent inter-bacterial killing assay of wild-type *V. cholerae* A1552 toward *E. coli* MC4100 (left) or *V. cholerae* A1552 harbouring the pVI2431 ΔA-VCA0117-His expression plasmid (right). The killing index was determined by measuring CFU/ml of each strain after co-cultivation under the indicated conditions as described in Experimental Procedures. Values are the average ± SE from three independent experiments. *P*-values *** ≤ 0.0001, ** ≤ 0.005 as determined by the Tukey–Kramer's test.

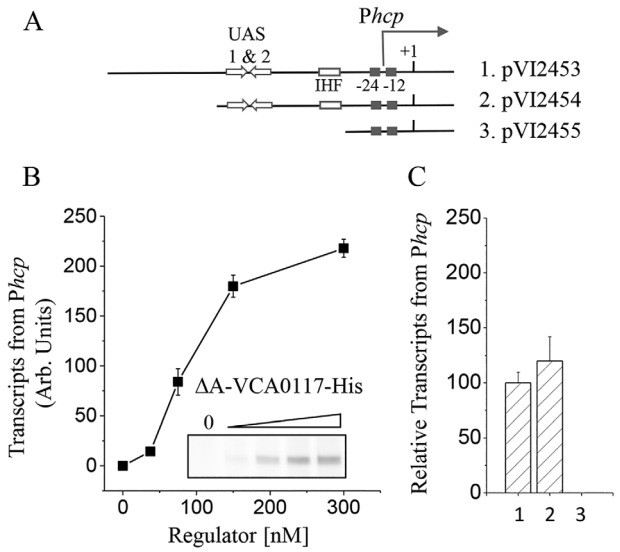

Fig 5. *In vitro* transcription by ΔA-VCA0117-His requires the UASs of IR3.

A. Schematic illustration of the σ^{54}-dependent P*hcp* promoter inserts carried by the *in vitro* transcription templates used in panels B and C. DNA sequences are as in Fig. 4.

B. Single-round *in vitro* transcription assays using 5 nM supercoiled plasmid DNA (template 1, pVI2453) in the presence of 20 nM σ^{54}-RNAP, 20 nM IHF, and increased concentrations of ΔA-VCA0117-His (0, 37.5, 75, 150 and 300 nM). Insert image is of the 300 nt transcript from one of two independent experiments used to obtain the graphed averages ± SE.

C. Single-round *in vitro* transcription assays using 5 nM of the indicated supercoiled DNA templates, which differ only in the extent of promoter upstream DNA. Promoter output was determined as under panel B in the presence of 100 nM ΔA-VCA0117-His. Graphed values are the averages of four independent experiments ± SE, and were normalised by setting the value with template 1 (pVI2453) as 100. *P*-values of template 1 and 2 as compared to template 3 are 0.00175 and 0.000490, respectively, as determined by the Tukey–Kramer's test.

these resides in the GAF-domain (either an Asp or a His residue at 116), while the other resides in the variable region between the centra1 C-domain and the DNA binding domain (either an Ala or a Thr residue at 449), see Supporting Information Fig. S1. We generated all combinations of these substitutions as His-tagged variants and tested their ability to promote transcription from the P*hcpII-luxAB* luciferase transcriptional reporter.

In *E. coli*, all four full length VCA0117-His variants were produced at similar levels and promoted similar output from the P*hcpII*, suggesting that all are equally active (Supporting Information Fig. S5). In comparison with ΔA-VCA0117-His, these full-length proteins are produced at much lower levels, leading to approximately 12%–15% of the transcription achieved with the more abundant ΔA-VCA0117-His derivative. In *V. cholerae* A1552, expression levels of the full-length VCA0117 variants were even lower, making comparisons using the luciferase reporter assays uninformative (Supporting Information Fig. S5). Therefore, we tested the ability of the four full-length VCA0117 proteins to promote transcription by monitoring Hcp production in *V. cholerae* A1552. As is the case in *E. coli*, all the full-length regulators again behaved similarly, promoting production of indistinguishable levels of Hcp that exceed those found with *V. cholerae* A1552 grown under non-inducing conditions (Fig. 7A).

The results in the Supporting Information Fig. S5 and Fig. 7A, demonstrate that the nature of residue 116 (Asp or a His) or residue 449 (Ala or Thr) does not influence the performance of VCA0117 in either *E. coli* or *V. cholerae*. To further explore the properties of full length VCA0117, we chose two variants – the $VCA0117_{A1552}$ protein (D116/A449) and its H116 counterpart – for *in vitro* analysis. Native proteins, purified from His-SUMO tagged variants as detailed in Experimental Procedures, were subject to single round *in vitro* transcription assays as described for ΔA-VCA0117-His. Consistent with *in vivo* data, both variants showed similar activities (Fig. 7B) and exhibited dose responses similar to ΔA-VCA0117-His (Fig. 5A). A side-by-side comparison of full-length VCA0117 proteins and the GAF-domain deleted ΔA-VCA0117-His suggest that at least under *in vitro* conditions, the full-length proteins have only a slightly lower (1.2- to 1.5-fold) ability to activate transcription by σ^{54}-RNA polymerase (Fig. 7C). As elaborated in the discussion, this is in marked contrast to other bEBPs where activities can be totally dependent on their N-terminal domains.

Conditional growth signals elevate endogenous VCA0117 levels in V. cholerae *A1552*

Specific transcriptional regulators are often present in cells at low abundance, ranging from tens to a few hundred molecules per cell (McAdams and Arkin, 1999; Ishihama *et al*., 2014). Given that Hcp levels were induced by ectopic expression of both ΔA-VCA0117-His (Fig. 1) and full-length VCA0117 variants (Fig. 7A), and that conditional growth signals elicit higher output from the P_{0107} promoter (Fig. 6A), it became of interest to determine if changes in endogenous VCA0117 levels could be detected in response to inducing conditions of low temperature and high osmolarity. For these experiments, we used antibodies raised against VCA0117.

Initial tests indicated that VCA0117 levels were too low to be detected in whole cell extracts (Supporting Information Fig. S6). Therefore, we performed quantitative Western analysis of extracts prepared from cells cultured as for the inter-bacterial killing assays (Fig. 6B) and compared endogenous VCA0117 expression levels with purified VCA0117 diluted in an extract from the *V. cholerae* VCA0117 null strain. Growth under fully inducing conditions elicited a 2- to 2.5-fold increase in VCA0117 levels as compared to growth under non-inducing conditions

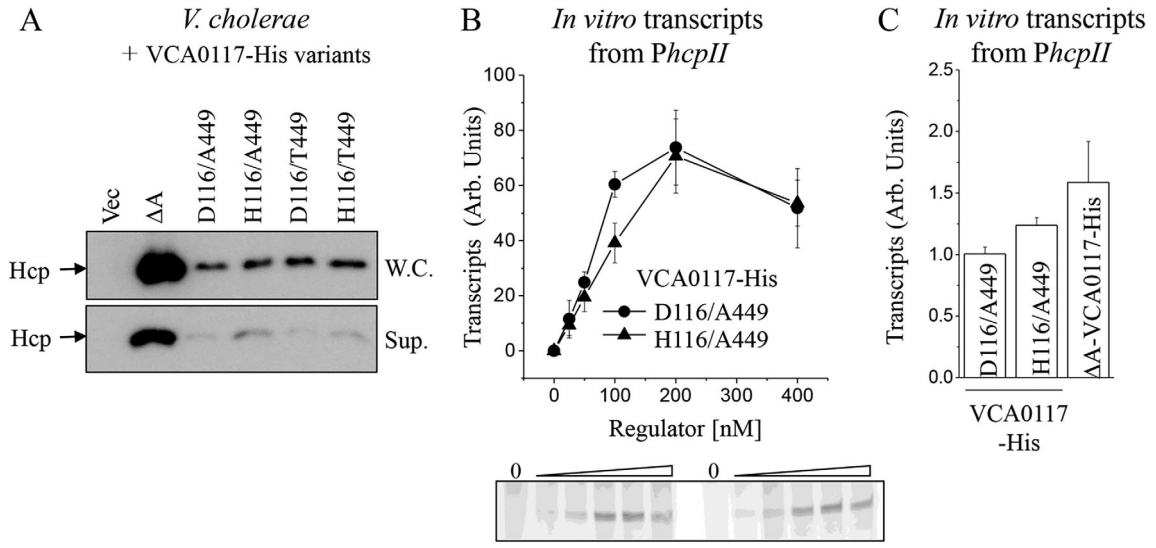

Fig 7. Full-length VCA0117 variants exhibit constitutive activity *in vitro*.
A. Immuno-detection of the 23 kDa Hcp protein in whole cell lysates (W.C.) and supernatants (Sup.) of *V. cholerae* A1552. Strains harbouring either a vector control (vec, pVI2403) or a P*tet* expression plasmid for the indicated VCA0117 derivative (ΔA, ΔA-VCA0117-His pVI2431; or full length VCA0117-His proteins pVI2431 to pVI2435), were cultured in LB under non-inducing conditions (37°C, 85 mM NaCl). Note that basal cellular Hcp levels in the vector control strain are not visible at the exposure shown. The cropped images are derived from samples analysed and processed on the same filter.
B. Single-round *in vitro* transcription assays with 5 nM supercoiled DNA templates harbouring the σ^{54}-dependent P*hcpII* promoter (pVI2453) in the presence of 20 nM σ^{54}-RNAP, 20 nM IHF, and increase concentrations of native *V. cholerae* A1552 VCA0117 (D116/A449) or its H116 counterpart (0, 25, 50, 100, 200 and 400 nM). Inset below is an image of the 300 nt transcript from one of two independent experiments used to obtain the graphed averages ± SE.
C. Single-round *in vitro* transcription assays as under panel B with 5 nM supercoiled DNA templates harbouring UAS1 and 2 (pVI2454) and 100 nM VCA0117 (D116/A449) or its H116 counterpart (H116/A449), or ΔA-VCA0117-His. Graphed values are averages ± SE of three or four independent determinations, normalised by setting the value with D116/A449 as 1. No statically significant difference between VCA0117 (D116/A449) or its H116 counterpart (H116/A449) (*P*-value = 0.55 as determined by the Tukey–Kramer's test).

(compare lanes 1 and 2 with 3 and 4, Fig. 8). As detailed in Experimental Procedures, the ~0.8 ng per 50 μg detected in extracts from non-inducing conditions corresponds to ~12–28 monomers per cell, while the ~2 ng levels of VCA0117 in extracts from inducing conditions corresponds to ~32–56 monomers per cell. As expanded upon in the discussion, these signal-responsive changes in VCA0117 levels suggest a threshold-level switch based on occupancy of the P*hcp* promoters. Consistent with this idea, analysis of extracts from other *V. cholerae* O1 El Tor strains (C6706 and N16961) showed even lower levels of this key regulatory protein that are likewise increased under inducing growth conditions (Supporting Information Fig. S7). In this respect, it is important to note that even the fully induced levels of VCA0117 in the A1552 strain are ~2 to 2.5-fold lower than those found in *V. cholerae* V52, which constitutively produces its T6SS machinery (Supporting Information Fig. S7 and data not shown).

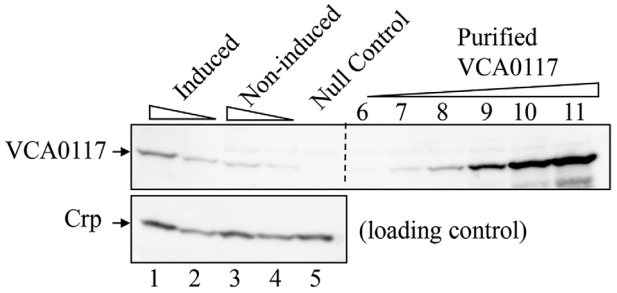

Fig 8. VCA0117 levels are elevated in response to high osmolarity and low temperature growth conditions.
Upper image: immuno-detection of endogenous VCA0117 using anti-VCA0117 antibodies: Lanes 1, 3 and 5 (50 μg), lanes 2, 4 (25 μg) of the indicated extracts; lanes 6–11 purified $VCA0117_{A1552}$ (0.12, 0.37, 1.1, 3.3, 10, 30 ng) prepared in 25 μg of extract from the *V. cholerae* A1552 VCA0117 null strain. *V. cholerae* A1552 was grown under full inducing conditions (23°C, 340 mM NaCl; lanes 1 and 2) or under non-inducing conditions (37°C, 85 mM NaCl; lanes 3and 4). Negative control extract from *V. cholerae* A1552 VCA0117 null is shown in lane 5. Lower image: immuno-detection of Crp (loading control) from the lower part of the same filter. The results shown are representative of three independent experiments.

Discussion

In *V. cholerae*, the bEBP VCA0117 is a critical regulator for expression of a functional T6SS since it is required for transcription of the genes encoding the structural components Hcp, and two of the three VgrG (1 and 2) proteins (Figs. 1–5). In *V. cholerae* O1 strains such as A1552,

expression of a functional T6SS apparatus is dependent on external signals of high osmolarity and low temperature prevalent in their natural aquatic habitat (Ishikawa *et al*., 2012, and Figs. 1B and 6B). Here, we traced this dependence to the activity of the P_{0107} promoter (Fig. 6A) that drives transcription of the large T6SS gene cluster within which VCA0117 is itself encoded. We found that ectopic expression of VCA0117 (Fig. 7A) or an active truncated variant (ΔA-VCA0117-His; Figs. 1B and 6B) is all that is needed to overcome the requirement for conditional growth signals for efficient production and secretion of Hcp and for T6SS-dependent inter-bacterial killing of *E. coli*. Furthermore, the conditional growth conditions that elicit efficient inter-bacterial killing by *V. cholerae* A1552 result in 2- to 2.5-fold elevation of the endogenous levels of this key regulator (Figs. 6 and 8).

The model that emerges from our data is that signal-integration at the level of P_{0107} activity results in elevated levels of the large operon-encoded components of the T6SS apparatus and VCA0117. Cascade regulation through elevation of VCA0117 levels, in turn, then mediates sufficient levels of transcription from the P*hcp* promoters to produce the Hcp and VgrG proteins required for completion of the functional system. Estimates from quantitative immune-detection of VCA0117 (Fig. 8) suggest that under non-inducing growth conditions (low osmolarity at 37°C) VCA0117 is present at ∼12–28 monomers per cell, which corresponds to the potential to form only 2 to 5 active hexamers. Signal-responsive induction (high osmolarity and growth at 23°C), and consequent 2- to 2.5-fold increase in the number of VCA0117 molecules per cell, likely tips the barrier needed to get sufficient active VCA0117 to occupy and promote transcription from the two P*hcp* promoters it regulates. Since Hcp is a critical component of the T6SS machinery, signal-responsive elevation of VCA0117 levels would result in signal-responsive production of Hcp and thereby signal-responsive secretion.

The pathoadaptive expression of T6SS in *V. cholerae* O1 strains (e.g. A1552) contrast the constitutively active T6SS of NOVC strains (e.g. V52) that expresses high levels of its VCA0117 counterpart (Supporting Information Fig. S7). Our analysis of VCA0117 variants that contain all combinations of the two amino acid residues that differ between these strains (D116/H116 and/or A449/T449) suggests that differential requirements for T6SS expression is not attributable to differences in the sequences of this key regulator, since they all behave similarly in both *in vivo* and *in vitro* transcription assays (Supporting Information Figs. S5 and Fig. 7). Rather, because the signals of low temperature and high osmolarity both independently and additively stimulate activity of the large operon promoter P_{0107} (Fig. 6A), it appears likely that these signals directly or indirectly control regulators of that promoter. Five potential candidates are (i) the IcIR-family osmoregulatory protein OscR, which is known to inhibit Hcp production in *V. cholerae* A1552 under low osmolarity conditions (Ishikawa *et al*., 2012); (ii) the lysR-like QstR protein that when overexpressed results in expression of a functional T6SS in this strain (Jaskolska *et al*., 2018); (iii) the response-regulator VxrB that likewise impacts expression of the T6SS of A1552 (Cheng *et al*., 2015); (iv) the global regulator TsrA, which coordinates with the quorum sensing system to repress expression of the T6SS of the *V. cholerae* O1 strain C6706 (Zheng *et al*., 2010); and (v) the TfoX and TfoY that differentially regulate T6SS performance in a variety of *Vibrio* species (Metzger *et al*., 2019).

Intriguingly, signal-responsive control of the P_{0107} promoter, whilst readily detected on solid media, could not be documented in liquid media (Supporting Information Fig. S4). Although molecular details are sparse, surface-sensing can be achieved by a variety of mechanisms that involve bacterial appendages such as the flagella and type IV pili (reviewed in the study by O'Toole and Wong, 2016). *Vibrio cholerae* has a single polar flagellum and a variety of pili, including type IV toxin co-regulated pili called TCP. Although highly speculative, our unanticipated finding that signal-responsive control requires growth on solid media may indicate a new level of regulation that involves cross-talk signalling from surface-attachment pili and/or friction-sensing flagella to co-ordinate expression (and therefore functioning) of the T6SS with surface contact.

As outlined above, control of the activity of the large operon promoter P_{0107} and otherwise limiting levels of VCA0117 appear as the key factor underlying signal-responsive control of the entire T6SS in *V. cholerae* A1552. VCA0117-mediated regulation of the P*hcp* promoters exhibits the hall-marks of many σ^{54}-dependent regulatory systems (Figs. 3–5, S1 and S5), including the obligate requirement for σ^{54} itself and high dependency on the DNA-bending protein IHF to such an extent that lack of either of these global regulatory molecules results in undetectable levels of Hcp (Fig. 3C). We found that VCA0117-mediated transcription of the P*hcp* promoters minimally requires the promoter proximal half of an imperfect inverted DNA repeat (IR3) to mediate detectable transcription from the P*hcp* promoters *in vivo* (Fig. 4). VCA0117 also relies on the presence of IR3 to mediate transcription *in vitro* (Fig. 5).

Because bEBPs need to be in a multimeric (usually hexameric) conformation in order to promote transcription, DNA binding can aid transcription by concentrating bEBPs in proximity of the promoters they control and even serves as an allosteric activator of some bEBPs (reviewed in the study by Bush and Dixon, 2012). The ability of VCA0117 to promote transcription through

binding to just one half of an inverted DNA repeat is not without precedence, since similar findings have been made with the *Pseudomonas putida* derived phenolic sensory bEBP DmpR (Sze *et al*., 2001). However, this ability contrasts the more demanding DNA binding requirement of other bEBPs. For example, the nitrogen oxide (NO) sensory NorR bEBP of *E. coli*, requires simultaneous binding to three inverted repeats to take up a hexameric conformation (Tucker *et al*., 2010; Bush *et al*., 2015).

Where present, the N-terminal A-domains of bEBPs usually serve to regulate the activity of bEBPs to either negatively and/or positively control their transcriptional promoting abilities (reviewed in the study by Shingler, 2011). Our *in silico* analysis suggests that the N-terminal A-domain of VCA0117 is a GAF-domain that is only found in bEBPs of aquatic bacteria of a limited number of orders of γ-proteobacteria, predominantly the *Vibrionales*, *Oceanospirillales* and *Alteromonadales*. Control functions of GAF-domains are usually modulated by direct sensing and binding of a ligand. For example, in the case of NifA of *Azotobacter vinelandii*, binding of 2-oxoglutarate by its GAF domain aids relief of inhibitory protein:protein interactions with NifL, releasing monomeric NifA to multimerise and thereby promote transcription (Little *et al*., 2000; Little and Dixon, 2003). In a similar manner, binding of formate to a GAF-domain within *E. coli* FhlA promotes multimerisation to its active form (Hopper *et al*., 1996; Self *et al*., 2001). In contrast, the GAF-domain of NorR serves at least two functions: repressing the correct interfacing of NorR with σ^{54}-RNA polymerase until NO is sensed through the non-haem iron of its GAF-domain (D'Autreaux *et al*., 2005; Tucker *et al*., 2010) and aiding binding and assembly of a hexamer through its three DNA binding sites (Tucker *et al*., 2010; Bush *et al*., 2015).

Irrespective of the type of ligand they respond to, deletion of regulatory GAF-domains generally results in variants with signal-independent activities (Leonhartsberger *et al*., 2000; Barrett *et al*., 2001; Tucker *et al*., 2010). Similarly, we found that a VCA0117 truncate lacking the GAF-domain likewise results in a derivative with constitutive transcriptional promoting ability both *in vivo* and *in vitro* (Figs. 1B and 5A). Differential expression levels of the full-length VCA0117-His proteins and the GAF deleted variant (ΔA-VCA0117-His) make it difficult to directly compare their relative activities *in vivo*; nevertheless, because full-length VCA0117 variants can promote transcription in *E. coli* (Supporting Information Fig. S5), they clearly do not require a *V. cholerae*-specific signal to be at least partially active. Indeed, native purified VCA0117 can efficiently promote transcription *in vitro*, albeit at modestly lower levels than that of the GAF-domain deleted derivative (Fig. 7C). We can envisage two reasons for the seemingly little difference in the *in vitro* activities. First, by analogy to NifA, the GAF-domain may play its major role during repressive protein: protein interactions that are not recapitulated in *E. coli* or our *in vitro* system. An alternative, and non-mutually exclusive, possibility is that the GAF-domain may have both inhibitory and stimulatory effects that are differentially amplified under the set conditions of *in vitro* assays. Such a possibility is suggested by the findings with FhlA, where high levels of ATP partially overcome the requirement for ligand-binding (Hopper *et al*., 1996). Our current dissection of VCA0117 should greatly facilitate future work to determine how the GAF-domain contributes to controlling the activity of this key regulator of *V. cholerae* T6SS.

Experimental Procedures

Bacterial strains and general procedures. Escherichia coli and *V. cholerae* A1552 strains are listed in the Supporting Information Table S1. Plasmids (Supporting Information Table S2) were constructed by standard molecular techniques as detailed in supplementary information. Liquid and agar-solidified Luria-Bertani/Lenox (LB) medium (AppliChem GmbH) was supplemented the following antibiotics for selection of chromosomal markers and/or maintenance of plasmids: carbenicillin (100 μg ml^{-1}), chloramphenicol (12.5 μg ml^{-1}), kanamycin (50 μg ml^{-1}), rifampicin (100 μg ml^{-1}), and streptomycin (50 μg ml^{-1}). Null derivatives of *V. cholerae* A1552 were constructed using double-site recombination as detailed in the Supporting Information.

Luciferase activity assays. For screening of promoter activities, individual colonies of *E. coli* or *V. cholerae* harbouring different transcriptional reporter systems were streaked on solid medium and incubated overnight at 37°C or 23°C as indicated. After addition of 100 μl of 1:1 diluted decanal (luciferase substrate) to the lid of inverted plates, light emission was recorded using a LAS 4000 imaging system (Fujifilm). Quantitative luciferase assays were performed on cultures grown and assayed at 30°C essentially as previously described (Sze *et al*., 1996). Overnight cultures were diluted 1:50 and pre-grown into exponential phase to ensure balanced growth prior to a second dilution to an OD_{600nm} of 0.05–0.08 and initiation of the experiment. Light emission from 100 μl of whole cells using a 1:2000 dilution of decanal was measured using an Infinite M200 (Tecan) illuminometer. Specific activity is expressed as relative luciferase units per OD_{600nm} of 1.0.

Purified proteins. Escherichia coli core RNA polymerase was purchased from Epicentre. Native *E. coli* σ^{54}

and IHF (integration host factor) were purified as previously described (Sze *et al*., 2001). Carboxy terminally His tagged ΔA-VCA0117-His was purified by Ni^{2+} affinity chromatography from a custom synthesised gene (GenScript, Supporting Information Fig. S8). For native full-length VCA0117 variants, P_{T7}-promoter expression plasmids for codon optimised versions of His-SUMO tagged VCA0117-D116/A449 (pVI2451) or VCA0117-H116/A449 (pVI2452) were constructed (Supporting Information Fig. S9). These plasmids were introduced into BL21(DE3) and protein expression was induced using auto-induction media (Studier, 2014) and culturing at 20°C. Proteins were purified from crude extracts at the Protein Expertise Platform (PEP) at Umeå University. Peak fractions from Ni^{2+} affinity purification were pooled and subject to gel filtration prior to overnight digestion with His tagged SUMO protease. After recovery of post-cleavage native proteins from the flow through from Ni^{2+} coupled resin, the proteins were concentrated (Centriprep R, molecular weight cut off 10 000; Merck). Buffer of the concentrated protein preparations were adjusted (final concentrations 28 mM Tris–HCl pH 8.0, 0.28 M NaCl, 0.14% Triton X Ultrapure, 0.056% Pluronic F-68, 1 mM EDTA, 0.56 mM β-mercaptoethanol, 50% glycerol) and then stored at −20°C until use.

In vitro *transcription assays.* Standard single-round *in vitro* assays (20 μl) were performed at 30°C in acetate buffer (AcB; 35 mM Tris-acetate pH 7.9, 5 mM magnesium acetate, 70 mM potassium acetate, 20 mM ammonium acetate, 1 mM DTT, 0.275 mg ml^{-1} bovine serum albumin) containing 3 mM ATP and 5 nM supercoiled DNA templates essentially as previously described (O'Neill *et al*., 2001). All DNA templates used were prepared by CsCl gradients, extensively dialyzed, and clarified through Micro Bio-Spin P30 columns (Bio-Rad) equilibrated with sterile H_2O to remove trace CsCl. Final reaction mixes additionally contained the indicated concentrations of VCA0117-variants, *E. coli* core RNA polymerase (20 nM), σ^{54} (100 nM), and IHF (20 nM). Core RNA polymerase and σ^{54} were pre-incubated for 5 min at 30°C to allow holoenzyme formation prior to addition of ATP, IHF and DNA template, followed by incubation for a further 10 min to allow closed-complex formation. Transcription was initiated by the addition of NTPs (final concentrations GTP, CTP, ATP 400 nM each; UTP 80 nM, 0.25 μl [α-^{32}P] UTP (Perkin Elmer) in the presence of heparin (0.1 mg ml^{-1}) to prevent re-initiation. Reactions were terminated after 10 min by the addition of 5 μl of a stop load mix (150 mM EDTA, 1 M NaCl, 14 M urea, 3% glycerol, 0.075% (w/v) xylene cyanol and 0.075% (w/v) bromophenol blue) and transcripts analysed on 7 M urea/5% (w/v) polyacrylamide sequencing gels. Radioactivity was quantified using a Typhoon FLA9500 imaging system (Molecular Dynamics).

Inter-bacterial killing assay. Functionality of the T6SS of *V. cholerae* was monitored, through the ability of *V. cholerae* to kill *E. coli* as previously described (Ishikawa *et al*., 2012). LB cultured *V. cholerae* A1552 and *E. coli* MC4100 were grown to an OD_{600nm} of 2.0 prior to mixing at a ratio of 1:1 (vol/vol). Forty microliters of this mixture was spotted onto pre-warmed LB agar plates with different NaCl concentrations (85 and 340 mM) and then incubated at 23°C or 37°C. After 4 h of incubation, bacterial cells were harvested from the plate and serial dilutions spread on LB plates with rifampicin or streptomycin to monitor the colony-forming ability (CFU ml^{-1}) of *V. cholerae* (predator) and *E. coli* (prey) respectively. In each experiment, the killing index was calculated from triplicate technical replicates by dividing the number of predator CFUs by the number of surviving prey CFUs.

Western analysis. Crude extracts from cells were prepared as follows. Cell pellets were washed and resupended in ice-cold sonication buffer (20 mM Tris–HCl pH 7.5, 0.2 mM NaCl, 1 mM EDTA) containing protease inhibitors (Complete EDTA-free protease inhibitor tablet; Roche) and disrupted by sonication. Extracts were clarified by centrifugation and total protein concentrations determined with PIERCE BCA protein assay (Thermo Scientific). To monitor Hcp protein production and secretion levels in *V. cholerae*, strains were cultured in LB medium containing either 85 mM or 340 mM NaCl to an OD_{600nm} of 2.0 and then processed as previously described (Ishikawa *et al*., 2012). Total whole cell protein (from the equivalent of 6.25 μl of culture) or secreted protein (equivalent to 250 μl of supernatant) were separated by SDS-PAGE and transferred to PVDF membranes for immune-detection.

Mouse monoclonal anti-Tetra His (QiaGen) and anti-σ^{54} (Neoclone) were used for detection of His tagged VCA0117 proteins and RpoN respectively. Purified rabbit polyclonal antibodies were used for detection of native proteins: anti-ΔA-VCA0117-His (Agrisera AB, this study) for detection of VCA0117, anti-Hcp for Hcp (Ishikawa *et al*., 2009), and anti-Crp as a control antibody (Balsalobre *et al*., 2006). HRP-conjugated donkey anti-rabbit (Agrisera AB) or goat anti-mouse (AbD Serotec) were used as secondary antibodies. Antibody decorated bands were detected using Clarity® Western ECL substrate (Bio Rad) or ECL™ Prime Western Blotting reagents (Amersham), while pre-stained protein molecular weight standards (SM0679, Fermentas) were used to determine the protein sizes.

Quantitative immune detection of endogenous VCA0117. Crude extracts were prepared from cells cultured in LB medium containing either 85 mM or 340 mM NaCl to an OD_{600nm} of 2.0 to 3.0 as for inter-bacterial killing assays. 50 and 25 μg of total soluble protein were resolved on 12% SDS gels to compare with purified VCA0117 (molecular mass 59 260) diluted in crude extracts from the *V. cholerae* VCA0117 null strain. Calculations of the number of VCA0117 cells were made in two independent ways. Based on the known size of *V. cholerae* (2 μm × 0.5 μm, cell volume 0.4 μm^3; Mizunoe *et al*., 1999) and 200 mg ml^{-1} concentration of *E. coli* cytosolic proteins (BioNumbers), 1 ng of VCA0117 in 50 μg is equivalent to ~16 momomers per cell. Based on OD_{600nm} correlations with colony forming units (OD 1 = 0.7×10^9 cells ml^{-1}; Ishikawa *et al*., 2012) and the total amount of protein extracted from a known volume of cells, 1 ng of VCA0117 in 50 μg is equivalent to ~35 momomers per cell.

In silico *analysis.* Full-length amino acid sequences of 239 proteins were retrieved from the NCBI RefSeq database, after having been identified using BLASTP (Altschul *et al*., 1990) and the first 154 residues of VCA0117 as the query. The sequences were aligned using MAFFT v7.164b (Katoh and Standley, 2013) with default parameters. The region of the alignment corresponding to residues 1–154 of VCA0117 was then extracted and used as input with HHPred (Söding *et al*., 2005) and MyHITS (Pagni *et al*., 2004), with the latter using the HMMER3 hmm search method of sensitive sequence searching (Eddy, 2011). Both analysis gave highly statistically significant hits to GAF domains of cyclic nucleotide binding/metabolising proteins (HHPred, probability >95%; MyHITS, <1e-04).

Statistical analysis. P-values for pair-wise comparisons of data sets (Fig. 3) were calculated with Welch's two-tailed *t*-test (Welch, 1947), while those for multi-data set comparisons (Figs. 4–7 and S5) were calculated using the Tukey–Kramer's (Tukey HSD) test (Kramer, 1956).

Acknowledgements

The authors thank Mikael Lindberg at the Protein Expertise Platform at Umeå University for native protein preparations, Kristina Hathazi for western analysis of *V. cholerae* RpoN levels and Vallo Varik for statistical analysis. This work was supported by The Kempe Foundations (SMK-1532 to KMA, JCK-1523 to HS; JCK-1728 to SNW), the Carl Tryggers Stiftelse (grant numbers CST15:337 to VS; CST13:488 to SNW) and The Swedish Research Council (grant numbers 2016-02047 and 2019-01024 to VS; 2013-2392 to SNW).

Authors Contributions

S.N.W. and V.S. conceived the study. H.S., K.M.A., T.I., M.G. and G.C.A. performed experiments and analysed data. T.I., A.S., and M.G. designed and generated purpose specific null strains and antibodies. H.S. and V.S. drafted the manuscript with input from all authors.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215**: 403–410.

Balsalobre, C., Johansson, J., and Uhlin, B.E. (2006) Cyclic AMP-dependent osmoregulation of *crp* gene expression in *Escherichia coli*. *J Bacteriol* **188**: 5935–5944.

Barrett, J., Ray, P., Sobczyk, A., Little, R., and Dixon, R. (2001) Concerted inhibition of the transcriptional activation functions of the enhancer-binding protein NIFA by the anti-activator NIFL. *Mol Microbiol* **39**: 480–493.

Barrios, H., Valderrama, B., and Morett, E. (1999) Compilation and analysis of σ^{54}-dependent promoter sequences. *Nucleic Acids Res* **27**: 4305–4313.

Basler, M. (2015) Type VI secretion system: secretion by a contractile nanomachine. *Philos Trans R Soc Lond B Biol Sci* **370**: 20150021.

Bernard, C.S., Brunet, Y.R., Gavioli, M., Lloubes, R., and Cascales, E. (2011) Regulation of type VI secretion gene clusters by σ^{54} and cognate enhancer binding proteins. *J Bacteriol* **193**: 2158–2167.

Bock, D., Medeiros, J.M., Tsao, H.F., Penz, T., Weiss, G.L., Aistleitner, K., *et al*. (2017) In situ architecture, function, and evolution of a contractile injection system. *Science* **357**: 713–717.

Burkinshaw, B.J., Liang, X., Wong, M., Le, A.N.H., Lam, L., and Dong, T.G. (2018) A type VI secretion system effector delivery mechanism dependent on PAAR and a chaperone-co-chaperone complex. *Nat Microbiol* **3**: 632–640.

Bush, M., and Dixon, R. (2012) The role of bacterial enhancer binding proteins as specialized activators of σ^{54}-dependent transcription. *Microbiol Mol Biol Rev* **76**: 497–529.

Bush, M., Ghosh, T., Sawicka, M., Moal, I.H., Bates, P.A., Dixon, R., and Zhang, X. (2015) The structural basis for enhancer-dependent assembly and activation of the AAA transcriptional activator NorR. *Mol Microbiol* **95**: 17–30.

Cases, I., Ussery, D.W., and de Lorenzo, V. (2003) The σ^{54} regulon (sigmulon) of *Pseudomonas putida*. *Environ Microbiol* **5**: 1281–1293.

Cheng, A.T., Ottemann, K.M., and Yildiz, F.H. (2015) *Vibrio cholerae* response regulator VxrB controls colonization and regulates the type VI secretion system. *PLoS Pathog* **11**: e1004933.

D'Autreaux, B., Tucker, N.P., Dixon, R., and Spiro, S. (2005) A non-haem iron centre in the transcription factor NorR senses nitric oxide. *Nature* **437**: 769–772.

Dong, T.G., and Mekalanos, J.J. (2012) Characterization of the RpoN regulon reveals differential regulation of T6SS

and new flagellar operons in *Vibrio cholerae* O37 strain V52. *Nucleic Acids Res* **40**: 7766–7775.
Eddy, S.R. (2011) Accelerated Profile HMM Searches. *PLoS Comput Biol* **7**: e1002195.
Goodrich, J.A., Schwartz, M.L., and McClure, W.R. (1990) Searching for and predicting the activity of sites for DNA binding proteins: compilation and analysis of the binding sites for *Escherichia coli* integration host factor (IHF). *Nucleic Acids Res* **18**: 4993–5000.
Hopper, S., Korsa, I., and Bock, A. (1996) The nucleotide concentration determines the specificity of *in vitro* transcription activation by the σ^{54}-dependent activator FhlA. *J Bacteriol* **178**: 199–203.
Ishihama, A., Kori, A., Koshio, E., Yamada, K., Maeda, H., Shimada, T., *et al*. (2014) Intracellular concentrations of 65 species of transcription factors with known regulatory functions in *Escherichia coli*. *J Bacteriol* **196**: 2718–2727.
Ishikawa, T., Rompikuntal, P.K., Lindmark, B., Milton, D.L., and Wai, S.N. (2009) Quorum sensing regulation of the two *hcp* alleles in *Vibrio cholerae* O1 strains. *PLoS One* **4**: e6734.
Ishikawa, T., Sabharwal, D., Broms, J., Milton, D.L., Sjostedt, A., Uhlin, B.E., and Wai, S.N. (2012) Pathoadaptive conditional regulation of the type VI secretion system in *Vibrio cholerae* O1 strains. *Infect Immun* **80**: 575–584.
Jaskolska, M., Stutzmann, S., Stoudmann, C., and Blokesch, M. (2018) QstR-dependent regulation of natural competence and type VI secretion in *Vibrio cholerae*. *Nucleic Acids Res* **46**: 10619–10634.
Joshi, A., Kostiuk, B., Rogers, A., Teschler, J., Pukatzki, S., and Yildiz, F.H. (2017) Rules of engagement: The type VI secretion system in *Vibrio cholerae*. *Trends Microbiol* **25**: 267–279.
Kaper, J.B., Morris, J.G., Jr., and Levine, M.M. (1995) Cholera. *Clin Microbiol Rev* **8**: 48–86.
Katoh, K., and Standley, D.M. (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* **30**: 772–780.
Kitaoka, M., Miyata, S.T., Brooks, T.M., Unterweger, D., and Pukatzki, S. (2011) VasH is a transcriptional regulator of the type VI secretion system functional in endemic and pandemic *Vibrio cholerae*. *J Bacteriol* **193**: 6471–6482.
Kostiuk, B., Unterweger, D., Provenzano, D., and Pukatzki, S. (2017) T6SS intraspecific competition orchestrates *Vibrio cholerae* genotypic diversity. *Int Microbiol* **20**: 130–137.
Kramer, C.Y. (1956) Extension of Multiple Range Tests to Group Means with Unequal Numbers of Replications. *Biometrics* **12**: 307–310.
Leonhartsberger, S., Ehrenreich, A., and Bock, A. (2000) Analysis of the domain structure and the DNA binding site of the transcriptional activator FhlA. *Eur J Biochem* **267**: 3672–3684.
Lien, Y.W., and Lai, E.M. (2017) Type VI secretion effectors: methodologies and biology. *Front Cell Infect Microbiol* **7**: 254.
Little, R., and Dixon, R. (2003) The amino-terminal GAF domain of *Azotobacter vinelandii* NifA binds 2-oxoglutarate to resist inhibition by NifL under nitrogen-limiting conditions. *J Biol Chem* **278**: 28711–28718.
Little, R., Reyes-Ramirez, F., Zhang, Y., van Heeswijk, W. C., and Dixon, R. (2000) Signal transduction to the *Azotobacter vinelandii* NIFL-NIFA regulatory system is influenced directly by interaction with 2-oxoglutarate and the PII regulatory protein. *EMBO J* **19**: 6041–6050.
Logan, S.L., Thomas, J., Yan, J., Baker, R.P., Shields, D.S., Xavier, J.B., *et al*. (2018) The *Vibrio cholerae* type VI secretion system can modulate host intestinal mechanics to displace gut bacterial symbionts. *Proc Natl Acad Sci U S A* **115**: E3779–E3787.
MacIntyre, D.L., Miyata, S.T., Kitaoka, M., and Pukatzki, S. (2010) The *Vibrio cholerae* type VI secretion system displays antimicrobial properties. *Proc Natl Acad Sci U S A* **107**: 19520–19524.
McAdams, H.H., and Arkin, A. (1999) It's a noisy business! Genetic regulation at the nanomolar scale. *Trends Genet* **15**: 65–69.
Metzger, L.C., Matthey, N., Stoudmann, C., Collas, E.J., and Blokesch, M. (2019) Ecological implications of gene regulation by TfoX and TfoY among diverse Vibrio species. *Environ Microbiol* **21**: 2231–2247.
Mizunoe, Y., Wai, S.N., Takade, A., and Yoshida, S.I. (1999) Isolation and characterization of rugose form of *Vibrio cholerae* O139 strain MO10. *Infect Immun* **67**: 958–963.
O'Neill, E., Wikstrom, P., and Shingler, V. (2001) An active role for a structured B-linker in effector control of the σ^{54}-dependent regulator DmpR. *EMBO J* **20**: 819–827.
O'Toole, G.A., and Wong, G.C. (2016) Sensational biofilms: surface sensing in bacteria. *Curr Opin Microbiol* **30**: 139–146.
Pagni, M., Ioannidis, V., Cerutti, L., Zahn-Zabal, M., Jongeneel, C.V., and Falquet, L. (2004) MyHits: a new interactive resource for protein annotation and domain identification. *Nucleic Acids Res* **32**: W332–W335.
Pukatzki, S., Ma, A.T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W.C., *et al*. (2006) Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. *Proc Natl Acad Sci U S A* **103**: 1528–1533.
Russell, A.B., Peterson, S.B., and Mougous, J.D. (2014) Type VI secretion system effectors: poisons with a purpose. *Nat Rev Microbiol* **12**: 137–148.
Self, W.T., Hasona, A., and Shanmugam, K.T. (2001) N-terminal truncations in the FhlA protein result in formate- and MoeA-independent expression of the *hyc* (formate hydrogenlyase) operon of *Escherichia coli*. *Microbiology* **147**: 3093–3104.
Shao, Y., and Bassler, B.L. (2014) Quorum regulatory small RNAs repress type VI secretion in *Vibrio cholerae*. *Mol Microbiol* **92**: 921–930.
Shingler, V. (2011) Signal sensory systems that impact σ^{54}-dependent transcription. *FEMS Microbiol Rev* **35**: 425–440.
Si, M., Wang, Y., Zhang, B., Zhao, C., Kang, Y., Bai, H., *et al*. (2017) The Type VI Secretion System Engages a Redox-Regulated Dual-Functional Heme Transporter for Zinc Acquisition. *Cell Rep* **20**: 949–959.
Söding, J., Biegert, A., and Lupas, A.N. (2005) The HHpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Res* **33**: W244–W248.

Studier, F.W. (2014) Stable expression clones and auto-induction for protein production in *E. coli*. *Methods Mol Biol* **1091**: 17–32.

Sze, C.C., Moore, T., and Shingler, V. (1996) Growth phase-dependent transcription of the σ^{54}-dependent Po promoter controlling the *Pseudomonas*-derived (methyl)phenol *dmp* operon of pVI150. *J Bacteriol* **178**: 3727–3735.

Sze, C.C., Laurie, A.D., and Shingler, V. (2001) *In vivo* and *in vitro* effects of integration host factor at the DmpR-regulated σ^{54}-dependent Po promoter. *J Bacteriol* **183**: 2842–2851.

Tucker, N.P., Ghosh, T., Bush, M., Zhang, X., and Dixon, R. (2010) Essential roles of three enhancer sites in σ^{54}-dependent transcription by the nitric oxide sensing regulatory protein NorR. *Nucleic Acids Res* **38**: 1182–1194.

Unterweger, D., Kitaoka, M., Miyata, S.T., Bachmann, V., Brooks, T.M., Moloney, J., *et al*. (2012) Constitutive type VI secretion system expression gives *Vibrio cholerae* intra- and interspecific competitive advantages. *PLoS One* **7**: e48320.

Vaitkevicius, K., Lindmark, B., Ou, G., Song, T., Toma, C., Iwanaga, M., *et al*. (2006) A *Vibrio cholerae* protease needed for killing of *Caenorhabditis elegans* has a role in protection from natural predator grazing. *Proc Natl Acad Sci U S A* **103**: 9280–9285.

Wang, T., Si, M., Song, Y., Zhu, W., Gao, F., Wang, Y., *et al*. (2015) Type VI Secretion System Transports Zn2+ to Combat Multiple Stresses and Host Immunity. *PLoS Pathog* **11**: e1005020.

Welch, B.L. (1947) The generalisation of student's problems when several different population variances are involved. *Biometrika* **34**: 28–35.

Zheng, J., Shin, O.S., Cameron, D.E., and Mekalanos, J.J. (2010) Quorum sensing and a global regulator TsrA control expression of type VI secretion and virulence in *Vibrio cholerae*. *Proc Natl Acad Sci U S A* **107**: 21128–21133.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supporting Information.