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The *Vibrio cholerae* VarS/VarA two-component system controls the expression of virulence proteins through ToxT regulation

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Although the conditions for inducing virulence protein expression *in vitro* are different, both classical and El Tor biotypes of *Vibrio cholerae* have been reported to regulate the expression of virulence proteins such as cholera toxin (CT) and toxin-coregulated pili (Tcp) through the ToxR/S/T system. The transcription activator ToxR responds to environmental stimuli such as pH and temperature and activates the second transcriptional regulator ToxT, which upregulates expression of virulence proteins. In addition to the ToxR/S/T signalling system, *V. cholerae* has been proposed to utilize another two-component system VarS/VarA to modulate expression of virulence genes. Previous study has shown that VarA of the VarS/VarA system is involved in the regulation of virulence proteins in the classical *V. cholerae* O395 strain; however, no further analysis was performed concerning VarS. In this study, we constructed *varS* mutants derived from the classical O395 and El Tor C6706 strains and demonstrated that VarS is also involved in the expression of the virulence proteins CT and Tcp from the *V. cholerae* classical and El Tor strains. This expression is through regulation of ToxT expression in response to environmental changes due to different toxin-inducing conditions.

INTRODUCTION

Cholera is an acutely dehydrating diarrhoeal disease caused by intestinal infection with the bacterium *Vibrio cholerae* (Faruque *et al.*, 1998). The two major disease-causing biotypes of *V. cholerae* are classical and El Tor and these biotypes exhibit differences in their epidemic nature and in the expression profiles of their virulence proteins, including cholera toxin (CT) and toxin-coregulated pili (Tcp) (Faruque *et al.*, 1998; Reidl & Klose, 2002). In humans, secretion of CT from *V. cholerae* results in elevated cAMP levels in intestinal epithelial cells and subsequent secretory diarrhoea (Lencer & Tsai, 2003; Spangler, 1992). Tcp is required for intestinal colonization (Reidl & Klose, 2002). Among these two biotypes, the classical strains are associated with more severe infection. One study showed that 11 % of infections with classical strains resulted in severe disease, whereas only 2 % of infections with El Tor strains resulted in a severe outcome (Kaper *et al.*, 1995). *In vitro*, different environments are required for production of virulence proteins. Classical strains produce CT and Tcp under ToxR-inducing conditions (moderate aeration at 30 °C in LB, pH 6.5 containing 85.5 mM NaCl) (Gardel & Mekalanos, 1996), whereas El Tor strains produce CT and Tcp under AKI conditions (cultivation for several hours in bicarbonate without aeration, followed by several hours of growth with vigorous aeration), which results in high-level expression of ToxR-regulated genes (DiRita *et al.*, 1996).

Although the conditions for virulence protein induction *in vitro* are different, expression of CT and Tcp in both strains has been reported to be controlled by two activator proteins, ToxR and ToxT, which operate in a cascade fashion with ToxR regulating the synthesis of ToxT (Fig. 1) (Higgins & DiRita, 1994). In the ToxR/S/T signalling circuit, the transcription activator ToxR, a transmembrane protein, responds to environmental stimuli such as pH and temperature by activating the second transcriptional regulator, ToxT, which in turn upregulates the expression of virulence proteins such as CT, Tcp and accessory colonization factor (Acf) (Fig. 1) (DiRita *et al.*, 1991).

†These authors contributed equally to this work.

Abbreviation: RT-PCR, reverse transcriptase PCR.

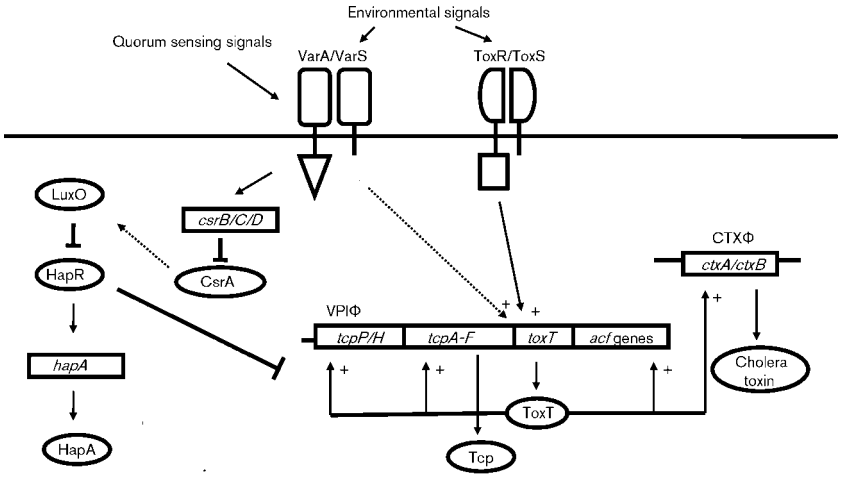

Fig. 1. Schematic representation of virulence regulation in *V. cholerae*. In addition to the ToxR/S system, the VarS/VarA system is involved in the expression of *toxT* in response to environmental signals such as pH and salt. The VarS/VarA system is also involved in the regulation of HapR through CsrB/C/D small RNA, CsrA and LuxO. Dotted lines denote hypothetical interactions. VPIΦ, *Vibrio* pathogenicity island phage; CTΦ, cholera toxin phage.

ToxT is a member of the AraC family of proteins and consists of two domains, an N-terminal dimerization and environment-sensing domain, and a C-terminal DNA-binding domain (Childers *et al.*, 2007). ToxT is also autoregulated, allowing for continuous expression of ToxT under favourable conditions (Yu & DiRita, 1999). ToxS stabilizes the conformation of ToxR in the periplasm. An additional pair of regulatory proteins, TcpP and TcpH, positively control the transcription of *toxT* (Carroll *et al.*, 1997; Häse & Mekalanos, 1998).

In addition to the ToxR/S/T signalling circuit, *V. cholerae* has been proposed to utilize another two-component family signalling system VarS/VarA to modulate expression of virulence genes in response to environmental signals (Lenz *et al.*, 2005; Wong *et al.*, 1998). In this system, the VarS protein is presumed to be a sensor kinase for the VarA response regulator. A previous study reported that a *varA* mutant, derived from the classical *V. cholerae* O395 strain, generated reduced levels of TcpA and CT (Wong *et al.*, 1998). ToxT seems to be involved in this signalling system in this mutant because ectopic overexpression of *toxT* in the *varA* mutant restored wild-type levels of CT production (Wong *et al.*, 1998). However, since the study by Wong *et al.* (1998), there has been no further analysis of VarS to our knowledge. In this study, to analyse the involvement of VarS in the regulation of virulence proteins in *V. cholerae*, we constructed *varS* mutant strains derived from the classical O395 and El Tor C6706 strains and analysed the expression of virulence factors such as CT and TcpA and the regulatory protein ToxT.

METHODS

Bacterial strains and plasmids. All strains and plasmids used in this study are described in Table 1. Control pBAD, pBAD-ToxT, pBAD-TcpPH and pJZ396 were kindly provided by Dr J. Zhu (University of Pennsylvania, USA). Strains were grown in Luria broth (LB) or on L agar (LA), and stored at –80 °C in LB containing 20 % glycerol (v/v). CT- and TcpA-inducing growth conditions were obtained for strain O395 by using a 1 : 1000 dilution of overnight culture into LB pH 6.5 (containing 85.5 mM NaCl) and growing at 30 °C, shaking at 250 r.p.m. (ToxR-inducing condition) (Gardel & Mekalanos, 1996). C6706 strain was grown in AKI medium as described previously (AKI condition) (DiRita *et al.*, 1996) when CT and TcpA production was required. Ampicillin (Amp) and streptomycin (Strep) were used at 100 μg ml^{-1} unless otherwise noted.

Nucleic acid manipulations. All nucleic acid manipulations were carried out according to standard protocols (Ausubel *et al.*, 1995). Cloning of PCR products was accomplished by using the TOPO TA Cloning kit (Invitrogen) in accordance with the manufacturer's directions. PCR primers were synthesized by either Qiagen or Bioneer. DNA sequencing was performed at the DNA sequencing facility at the Korea National Institute of Health (Seoul, Korea). PCRs (50 μl total) were typically performed using ex*Taq* polymerase under conditions specified by the manufacturer (TaKaRa).

Construction of *V. cholerae* VarS and VarA mutants. For the construction of pCVD-VarS2, the coding region of *varS* was amplified by PCR from *V. cholerae* genomic DNA using the oligonucleotides 5′-CATATGACTCAAAGATATGGCTTGCGCGCC-3′, carrying the *varS* coding sequence from nt 1 to 24 and containing an *Nde*I site (underlined) (Heidelberg *et al.*, 2000), and 5′-TCTAGATCAGTTCAGATAGTCGCGAGAGGC-3′, complementary to nt 2761–2784 of the *varS* coding sequence and containing an *Xba*I site (underlined). The PCR product was cloned into the pCRII-TOPO vector to produce pCRII-VarS1. pCRII-VarS1 was digested with *Acc*I to remove 1.6 kb of the *varS* coding region, producing pCRII-VarS2. Then, pCRII-VarS2 was digested with *Sac*I and *Xba*I and introduced between the *Sac*I and *Xba*I sites of pCVD442 (Donnenberg & Kaper, 1991) to generate pCVD-VarS2.

For the construction of pCVD-VarA2, the coding region of *varA* was amplified by PCR from the *V. cholerae* genomic DNA using oligonucleotides 5′-GAGCTCGTGCAGAGCCAATAGATAAGTGTGGA-3′, carrying the sequences from nt 1 to 26 and containing a *Sac*I site (underlined) (Heidelberg *et al.*, 2000), and 5′-TCTAGAGTCGAGATTCTTGCGAAAATAGCT-3′, complementary to nt 812–824 of the *varA* coding sequence and containing an *Xba*I site (underlined). The PCR product was cloned into pCRII-TOPO vector to produce pCRII-VarA1. pCRII-VarA1 was digested with *Aat*II and *Sal*I to remove 0.6 kb of the coding region, producing pCRII-VarA2. Then, pCRII-VarA2 was digested with *Sac*I and *Xba*I and introduced into pCVD441 (Donnenberg & Kaper, 1991) to generate pCVD-VarA2.

To disrupt the *varS* or *varA* gene in *V. cholerae*, pCVD-VarS2 or pCVD-VarA2 was transformed into *E. coli* SM10/λ *pir*$^{+}$ (Simon *et al.*,

Table 1. Strains, plasmids and primers used in this study

Strain, plasmid or primer	Description	Reference or source
***E. coli* strains**		
DH5α/λ*pir*	λ *pir* lysogen of DH5**α** αα	Laboratory collection
SM10/λ*pir*	*thi thr leu tonA lacY supE recA*::RP-4-Tc::Mu (λ*pir*) R6K	Laboratory collection (Simon *et al.*, 1983)
TOP10	F⁻ *mrcA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80 *lac*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (*strR*) *endA1 nupG*	Invitrogen
***V. cholerae* strains**		
O395	Strep^r (Classical, Ogawa, wild-type)	Laboratory collection
O395-VS	O395 Δ*varS*	Present study
C6706	Strep^r (E1 Tor, Inaba, wild-type)	Laboratory collection
C6706-VS	C6706 Δ*varS*	Present study
C6706-VA	C6706 Δ*varA*	Present study
Plasmids		
pCVD442	Suicide vector (*oriR6K mobRP4 sacB*, Amp^r)	Laboratory collection (Donnenberg & Kaper, 1991)
pCVD-VarS2	*Sac*I–*Xba*I Δ*varS* gene fragment cloned in pCVD442	Laboratory collection (Jang *et al.*, 2010)
pCVD-VarA2	*Sac*I–*Xba*I Δ*varA* gene fragment cloned in pCVD442	Present study
pMal-VarS	2.7 kb *Nde*I/*Xba*I fragment from pCVD-VarS2 cloned into pMal-c2X	Present study
RT-PCR primers	**Primer sequence 5′→3′**	**Target gene in *V. cholerae***
CTB1058	GATTTGTGTGCAGAATACCACAACAC	*ctxB*
CTB1259	CCTCAGGGTATCCTTCATCCTTTC	
RecA1061	CGCTTTACCTTGGCCGATTT	*recA*
RecA578	GTGCTGTGGATGTCATCGTTGTTG	
TcpA2053	GTGGTCTCAGCGGGTGTTGTT	*tcpA*
TcpA3060	CAGCGCCAGTAGCAGCATCT	

1983) and then transferred into *V. cholerae* strains by conjugation. Recombinants were selected on Amp- and Strep-containing plates. To select for a second recombination event, the recombinants were grown overnight on LA at 37 °C without selection and plated on LA (without NaCl) containing 6 % sucrose and Strep, thus selecting for sucrose-resistant, Amp-sensitive colonies. The disruption of *varS* or *varA* in *V. cholerae* was confirmed by PCR amplification and sequencing.

To construct pMAL-VarS, the 2.7 kb *Nde*I/*Xba*I fragment from pCRII-VarS1 was cloned into the *Nde*I/*Xba*I site of pMAL-c2X (New England Biolabs) to generate pMAL-VarS.

CT production assay. *V. cholerae* cells were grown in appropriate conditions as indicated. Then, culture supernatants and cells were collected by centrifugation. Cells were washed with PBS, broken by sonication and used for Western blotting. CT from the supernatant was quantified with ganglioside-dependent ELISA, as described previously, with commercial CT subunit (Sigma) as a standard (Gardel & Mekalanos, 1994).

Western blot analysis. Whole-cell lysates were prepared in bacterial protein extraction solution (Intron) and protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce). Equal amounts of protein were separated on 12 % NuPage Bistris gel (Invitrogen), transferred to nitrocellulose membranes (Amersham Biosciences). After transfer, the blots were blocked with 5 % skimmed milk in Tris-buffered saline containing 0.05 % Tween 20 (TBS-T) for 1 h at room temperature and then incubated overnight at 4 °C with polyclonal anti-TcpA antibody at 1 : 1000 dilutions in 5 % skimmed milk in TBS-T. Anti-TcpA antibody was kindly provided by Dr M. K. Waldor (Harvard Medical School, USA). The blots were then washed three times for 10 min with TBS-T and incubated for 1 h at room temperature with horseradish peroxidase-conjugated polyclonal anti-rabbit IgG (Jackson Immunoresearch) diluted 1 : 2000 in 5 % skimmed milk in TBS-T. The blots were washed three times with TBS-T. The blots were visualised using a West-one detection system (Intron).

Luciferase assay. The luciferase activity was assayed using the Promega luciferase assay system according to the manufacturer's instructions with a Victoe3 (Perkin Elmer) plate reader. All luciferase activity measurements were normalized to the protein concentration.

Reverse-transcriptase PCR (RT-PCR) analysis. Total RNA was isolated from *V. cholerae* using the RNeasy mini kit (Qiagen). Reverse transcription was performed using the Superscript III first-stand synthesis system (Invitrogen) according to the manufacturer's protocol. Briefly, 2 μg total RNA was used for the reverse transcription reaction. The synthesized cDNA was subjected to RT-PCR amplification using the premix-PCR kit (Bioneer) and then separated using agarose gel electrophoresis. The primers used for RT-PCR amplification are listed in Table 1. *recA* mRNA was analysed as a reference. Signal densities for amplification products were quantified by densitometry (Alpha Innotech) after normalizing to the reference.

Infant mouse colonization assay. Competition assays for intestinal colonization were performed as described previously (Zhu *et al.*, 2002). Briefly, *V. cholerae* mutant strains (Lac$^+$) were mixed with the wild-type strain (Lac$^-$), and approximately 10^5 cells were inoculated into 4- to 5-day-old CD-1 suckling mice. After 20 h, intestinal homogenates were collected and the ratio of mutant to wild-type

bacteria was determined by plating homogenates on LA containing X-Gal and Strep. The *in vivo* competitive index was calculated by dividing the mutant : wild-type ratio recovered from the small intestine by the inoculum input ratio.

Azocasein and biofilm assays. Azocasein assays were performed as described previously (Zhu *et al.*, 2002). For biofilm assays, overnight cultures of *V. cholerae* were inoculated at a 1 : 100 dilution into LB and incubated in borosilicate tubes for 18 h at 22 °C. Subsequently, the tubes were rinsed with distilled water and then filled with crystal violet stain. After 5 min, the tubes were rinsed. The biofilm-associated crystal violet was resuspended in DMSO, and the OD_{570} of the resulting suspension was measured.

RESULTS AND DISCUSSION

V. cholerae O395-VS and C6706-VS exhibit decreased CT and TcpA production

varS mutants O395-VS and C6706-VS, derived from *V. cholerae* O395 and C6706, respectively, were produced using pCVD-VarS2 (Jang *et al.*, 2010), as described in Methods, and correct deletion was confirmed by PCR and sequencing (data not shown). To confirm the role of VarS in the production of *V. cholerae* virulence proteins, TcpA and CT expression in the wild-type and *varS* mutants was analysed by Western blotting and ganglioside-dependent ELISA (Gardel & Mekalanos, 1994), respectively. O395 and O395-VS were grown under ToxR-inducing conditions (Gardel & Mekalanos, 1996) whereas C6706 and C6706-VS were grown under AKI conditions (DiRita *et al.*, 1996). The expression of TcpA was downregulated in the *varS* mutants (Fig. 2a, b). O395-VS and C6706-VS exhibited approximately 134- and 2.5-fold reduction in CT expression relative to the wild-type, respectively (Fig. 2a, b). In addition, autoagglutination of O395-VS under ToxR-inducing conditions, which requires expression of functional TcpA, was not observed (data not shown). The expression of TcpA and CT in the *varS* mutants was recovered to nearly wild-type levels by transformation of pMal-VarS with induction using 1 mM IPTG

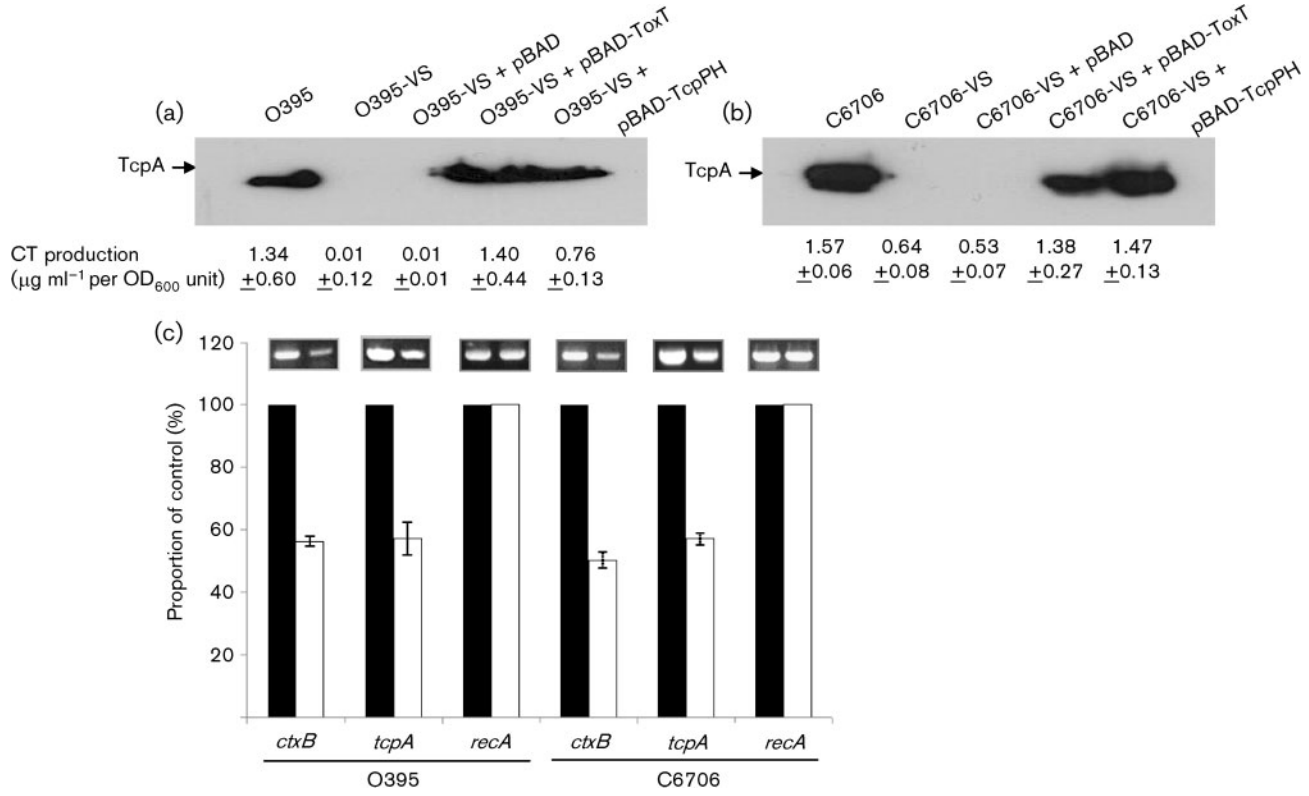

Fig. 2. (a, b) Expression of TcpA in *V. cholerae* O395 (a) and C6706 (b) wild-type and *varS* mutants and in *varS* mutants transformed by electroporation with pBAD (vector alone), pBAD-ToxT or pBAD-TcpPH. Strains were grown under ToxR-inducing conditions (for O395 strains) or AKI conditions (for C6706 strains) in the presence of 0.1 % arabinose, and cell pellets were subjected to Western blot analysis using anti-TcpA antibody (a gift from Dr M. K. Waldor, Harvard Medical School, USA). The corresponding cell-free culture supernatants were assayed for CT production using ganglioside-dependent ELISA; values are indicated below each blot. (c) Expression of *ctxB* and *tcpA* genes were tested using RT-PCR. Total RNA (2 μg) isolated from O395 and C6706 wild-type (black columns) and *varS* mutant (white columns) strains was subjected to RT-PCR analysis using the primers described in Table 1. *recA* mRNA was analysed as an internal control. Amplification products were separated by agarose gel electrophoresis, photographed (inset) and quantified. Data represent the means ± SD from three independent reactions.

(Supplementary Fig. S1a, b, available with the online version of this paper). Decreased *ctxB* and *tcpA* expression was also confirmed by RT-PCR analysis of total RNA isolated from cultures of each mutant (Fig. 2c). The expression of *ctxB* and *tcpA* genes was decreased by 1.99- and 1.75-fold in O395-VS and by 1.75- and 1.72-fold in C6706-VS, respectively, compared with the wild-types. *varA* expression remained constant in all strains, whereas *varS* expression was not detected in O395-VS and C6706-VS (data not shown). The gene encoding RecA, which did not show an appreciable change in expression level during growth was used as a control. As previously reported in the case of the VarA mutant of O395 (Wong *et al.*, 1998), the C6706 VarA mutant showed a similar phenotype to C6706-VS and decreased expression of TcpA and CT (Supplementary Fig. S1c).

toxT expression is decreased in O395-VS and C6706-VS mutants and ectopic ToxT or TcpPH expression restores the ability to produce CT and TcpA

Previous data suggest that in O395, VarA of the VarS/VarA system is involved in ToxT expression in a ToxR-independent manner, acting downstream of ToxR, and VarA does not regulate the expression of ToxR (Wong *et al.*, 1998). Thus, we examined *toxT* expression in the *varS* mutants under their relevant inducing conditions using RT-PCR (Fig. 3a). Expression of *toxT* was 1.9- and 3.1-fold lower in O395-VS and C6706-VS, respectively, than in the wild-type (Fig. 3a). RecA gene expression was analysed as an internal control. In addition, *toxT* promoter activity was examined by measuring luciferase activity in *varS* mutants transformed with pJZ396, which contains the *toxT* promoter sequence driving luciferase gene (*luxDCABE*) expression. As shown in Fig. 3(b, c), luciferase activity in the O395 and C6706 *varS* mutant strains containing pJZ396 was 10.4- and 2.4-fold lower, respectively, under inducing conditions than in the wild-type containing pJZ396. Strains without pJZ396 did not exhibit luciferase activity (Fig. 3b, c).

Since ToxT expression was reduced in *varS* mutants, we determined whether the overexpression of ToxT or TcpPH could rescue *varS* mutant phenotypes to confirm the role of VarS/VarA in the downstream pathway of ToxR (Wong *et al.*, 1998). ToxT can activate *tcpA* and *ctxAB* promoters (DiRita *et al.*, 1991) and ToxT can also autoregulate itself (Yu & DiRita, 1999). TcpPH has been known to activate the *toxT* promoter (Häse & Mekalanos, 1998). TcpPH from an inducible promoter could activate a *toxT*::*lacZ* reporter product construct independent of ToxR (Häse & Mekalanos, 1998). When the pBAD-ToxT plasmid carrying *toxT* under the control of the arabinose-inducible promoter was introduced into strains O395-VS and C6706-VS, TcpA expression in the presence of 0.1 % arabinose was restored to almost wild-type levels (Fig. 2a, b). Similarly, CT expression in the mutants containing pBAD-ToxT was

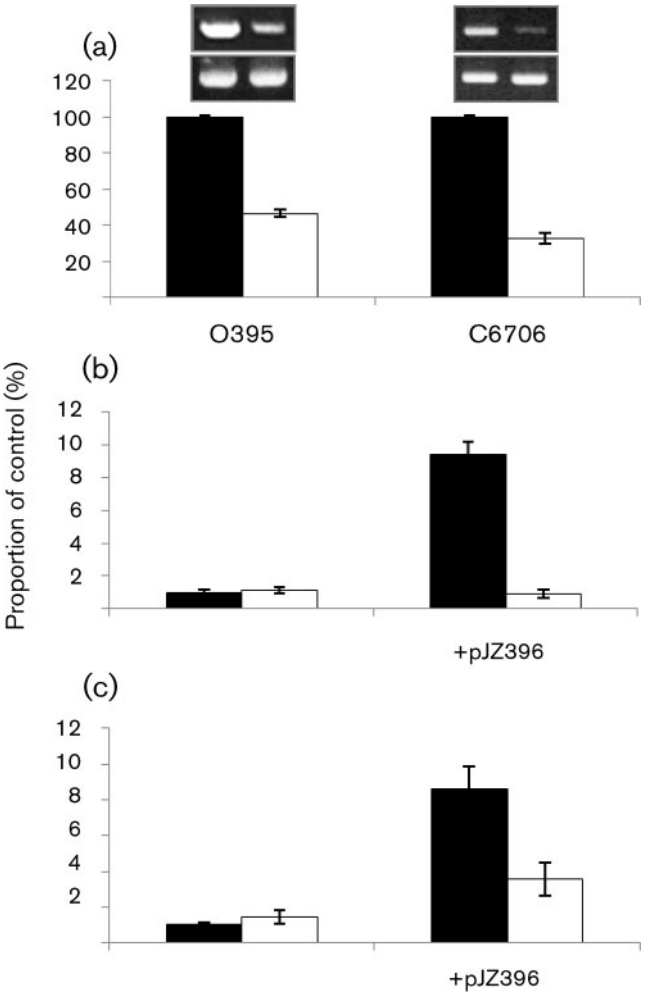

Fig. 3. VarS regulation of *toxT* expression in *V. cholerae* wild-type and *varS* mutants. (a) To examine *toxT* expression, total RNA (2 μg) isolated from O395 and C6706 wild-type (black columns) and *varS* mutant (white columns) strains was subjected to RT-PCR analysis using the primers described in Table 1. *recA* mRNA was analysed as an internal control (lower inset). Amplification products were separated by agarose gel electrophoresis, photographed (inset) and quantified. Data represent the means ± SD from three independent reactions. (b, c) To examine *toxT* promoter activity, untransformed *V. cholerae* wild-type and *varS* mutants or those transformed with the ToxT luciferase reporter pJZ396 were grown under ToxR-inducing conditions (O395 and O395-VS; b) or under AKI conditions (C6706 and C6706-VS; c) and assayed for luciferase activity. Data reflect mean (± SD) relative luciferase activity in wild-type (black columns) and mutant (white columns) strains from three independent experiments.

104.5 and 87.9 %, respectively, of that in the wild-type in the presence of 0.1 % arabinose (Fig. 2a, b). Overexpression of TcpPH from an inducible promoter in O395-VS and C6706-VS restored TcpA expression to nearly wild-type levels and decreased CT production by 56.7 and 93.6 %, respectively, compared with the wild-types (Fig. 2a, b). Our

RT-PCR experiment in both wild-types and *varS* mutants showed that VarS did not regulate the expression of *toxR* (data not shown). These observations indicate that VarS regulates the expression of *V. cholerae* virulence proteins, including CT and Tcp, via transcriptional regulation of ToxT, probably independent of ToxR (Fig. 1).

V. cholerae O395-VS and C6706-VS exhibit decreased colonization ability in infant mice

To assess the role of VarS in bacterial colonization, we performed an *in vivo* colonization assay using the infant mouse model (Zhu *et al.*, 2002). We expected decreased TcpA expression due to reduced *toxT* expression in the *varS* mutants to result in decreased *V. cholerae* colonization. As expected, O395 and C6706 exhibited competitive indices of 1.49 ± 0.21 and 0.98 ± 0.25, respectively, whereas O395-VS and C6706-VS exhibited competitive indices of 0.03 ± 0.04 and below the limit of detection, respectively (Table 2). These results confirm that VarS is important for the expression of *V. cholerae* virulence determinants, including TcpA, that are necessary for efficient colonization, and that the VarS/VarA regulatory circuit not only regulates the expression of TcpA and the production of CT *in vitro* but is also likely to regulate expression of TcpA *in vivo*.

VarS/VarA system homologues exist in a variety of Gram-negative bacteria, including *Pseudomonas aeruginosa* (GacS/GacA), and have been reported to be involved in the expression of virulence proteins (Rahme *et al.*, 1997; Reimmann *et al.*, 1997). In the CHA0 strain of *Pseudomonas fluorescens*, the VarA response regulator homologue, GacA, is essential for the synthesis of the extracellular protease (AprA) and secondary metabolites including hydrogen cyanide, and was found to control the hydrogen cyanide biosynthesis genes (*hcnABC*) and the *aprA* gene indirectly via a post-transcriptional mechanism (Blumer *et al.*, 1999). A distinct recognition motif [$(Py)_nCA.GGA$] that overlaps the ribosome-binding site appears essential for GacA-mediated regulation (Fig. 4);

Table 2. Competitive index of *V. cholerae* O395 and C6706 *varS* mutants

Strain	Genotype	Inoculated mice (*n*)	Competitive index*
O395	WT	7	1.49 ± 0.21
O395-VS	O395 Δ*varS*	9	0.03 ± 0.04
C6706	WT	4	0.98 ± 0.25
C6706-VS	C6706 Δ*varS*	7	<limit of detection

*Competitive index is defined as the output ratio of mutant : wild-type bacteria divided by the input ratio of mutant : wild-type bacteria; values reflect the means ± SD for the number of mice indicated. All *in vivo* competitions were performed using Lac$^-$ derivatives of O395 and C6706 as competing strains.

Fig. 4. Alignment of the regions containing the ribosome-binding site of *hcnA* and *aprA* genes of *P. fluorescens* CHA0, and *toxT* genes of *V. cholerae* O395 and C6706. In each case, translation is initiated at the ATG codon shown at the 3′ end.

substitution or insertion of a single strategically located nucleotide in the *hcn* leader mRNA of *P. fluorescens* completely eliminated GacA-mediated control (Blumer *et al.*, 1999). Based on these results, we investigated whether the sequence around the *toxT* promoter ribosome-binding site is homologous to that of the ribosome-binding site from *P. fluorescens*, and found that some important conserved nucleotides were substituted into different nucleotides (Fig. 4). This observation suggests that in *V. cholerae*, VarA might not regulate the expression of virulence genes at a post-transcriptional level. Wong *et al.* (1998) suggested that VarA modulates CT production and TcpA expression in a ToxR-independent manner, most likely acting upstream of the *toxT* promoter. We are currently examining the mechanism of VarA regulation of virulence protein expression in *V. cholerae*.

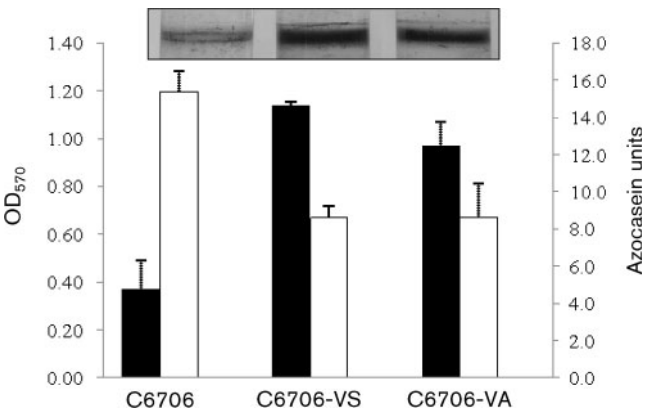

Fig. 5. Biofilm production is increased while HA protease production is decreased in C6706-VS and C6706-VA mutants. A comparison of biofilms produced by wild-type C6706, C6706-VS and C6706-VA mutants. The photograph (inset) shows crystal violet staining in borosilicate tubes containing C6706, C6706-VS or C6706-VA. The normalized data are presented for these assays (black columns). The OD_{570} values are a measure of crystal violet staining, which is proportional to level of biofilm formation. HA protease production is shown for C6706 wild-type and mutant strains (white columns). Overnight cultures were diluted 1 : 100 in LB and incubated at 37 °C. Samples were taken at 8 h to determine the azocasein activity. One azocasein unit is defined as the amount of enzyme producing an increase of 0.01 OD unit per h. Data represent the means ± SD from three independent experiments.

Previously, the CAI-1-CqsS and AI-2 LuxPQ quorum-sensing systems have been reported to regulate the expression of virulence factors (Hammer & Bassler, 2003; Lenz *et al.*, 2004; Chen *et al.*, 2002). The former system is composed of the CAI-1 autoinducer of unknown structure and CqsS, its two-component sensor. The latter system is made up of AI-2 (a furanosyl borate diester), the periplasmic binding protein LuxP, and the two component sensor LuxQ. At low cell density, in the absence of autoinducers, the sensors act as a kinase and transfer phosphate via LuxU to LuxO, resulting in activation of LuxO (Hammer & Bassler, 2003; Lenz *et al.*, 2004). Recently, the VarS/VarA–CsrA/B/C/D system was also reported to control the LuxO response regulator as a third sensory system (Fig. 1) (Lenz *et al.*, 2005; Lenz & Bassler, 2007). Although the signal for the pathway is not clear, it was proposed that at a low cell density, the VarS sensor kinase is inactive and does not phosphorylate the response regulator VarA. Unphosphorylated VarA is also inactive and therefore does not activate transcription of genes encoding the CsrB/C/D small RNAs (sRNAs). As a consequence, CsrA, a post-transcriptional regulator, is free to activate LuxO, which then destabilizes the mRNA encoding HapR, the master-regulator of quorum sensing in *V. cholerae* (Lenz *et al.*, 2005; Lenz & Bassler, 2007). At high cell density, VarS phosphorylates VarA. Phosphorylated VarA activates the genes encoding the CsrB/C/D sRNAs. These sRNAs then bind CsrA, sequestering CsrA from its targets. This leads to diminished LuxO activity, which in turn enhances HapR expression (Lenz *et al.*, 2005; Lenz & Bassler, 2007). A high concentration of HapR represses genes such as those encoding the virulence factors CT and Tcp and those required for biofilm production, while enhancing expression of genes including haemagglutinin (HA) protease (Jang *et al.*, 2010; Jobling & Holmes, 1997; Zhu *et al.*, 2002) (Fig. 1). In accordance with this previously proposed model, in our study, biofilm formation was increased in VarS and VarA mutants of C6706, which were locked in low cell density mode due to inactivation of VarS or VarA protein (resulting in blocking of further signalling), by 3.1- and 2.6-fold compared with wild-type, respectively, while HA protease activities were decreased in both mutants by 56.1 and 55.9 % compared with the wild-type, respectively (Fig. 5). Together with these previous studies, our results indicate that in *V. cholerae*, the VarS/VarA system plays an important role in the regulation of virulence protein expression via both quorum-sensing through HapR and environmental stimuli through ToxT.

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