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A branch in the ToxR regulatory cascade of *Vibrio cholerae* revealed by characterization of *toxT* mutant strains

Gretchen A. Champion,[1†] Melody N. Neely,[1,2] Molly A. Brennan[3] and Victor J. DiRita[1,2,3*]

[1]Department of Microbiology and Immunology, [2]Cellular Biotechnology Program, and [3]Unit for Laboratory Animal Medicine, University of Michigan Medical School, Ann Arbor, Michigan 48108-0620, USA.

Summary

Co-ordinate expression of genes associated with pathogenicity in *Vibrio cholerae* requires two transcription activators, ToxR and ToxT. Work carried out to date suggests that ToxR activates transcription of the *toxT* gene and that ToxT directly activates transcription of several genes whose products play a role in colonization or CT production by *V. cholerae*. Previous work also suggests that ToxR can directly activate transcription of the CT operon (*ctxAB*) independently of ToxT, thereby implying a degree of complexity in control of the *ctxAB* operon not found with other genes of the ToxR regulon. We tested the regulatory cascade model of virulence gene expression by constructing strains of classical and El Tor *V. cholerae* deleted for the coding sequence for the putative DNA-binding domain of *toxT*. Phenotypic analysis of these strains suggests that *V. cholerae* has ToxT-dependent and ToxT-independent branches of its virulence regulon. The results also raise questions about the precise role for ToxR in activation of *ctxAB* transcription.

Introduction

Vibrio cholerae causes cholera in humans. The primary virulence factor of this Gram-negative organism is the cholera toxin (CT), an ADP-ribosylating toxin encoded by the *ctxAB* operon, which resides on the genome of a filamentous, lysogenic phage called CTXΦ (Waldor and Mekalanos, 1996). Other factors are associated with pathogenicity of *V. cholerae* and their expression is co-ordinate with that of CT (Taylor *et al*., 1987; Peterson and Mekalanos, 1988). The genes encoding toxin and other virulence factors are all under the control of a regulatory protein called ToxR, and are collectively termed the ToxR regulon (Miller and Mekalanos, 1988; Peterson and Mekalanos, 1988). Two of these factors are the toxin co-regulated pilus (TCP) and the accessory colonization factor (ACF), the genes for which are physically linked on the *V. cholerae* chromosome (Everiss *et al*., 1996). Expression of a major outer membrane protein, OmpU, is also co-ordinate with expression of CT and a second outer membrane protein, OmpT, is expressed under conditions in which OmpU and the other toxin co-regulated genes are not expressed. The roles of OmpU and OmpT in pathogenicity of *V. cholerae* have not been established, but OmpU may be an adhesin (Sperandio *et al*., 1995; Sperandio *et al*., 1996). The gene for aldehyde dehydrogenase in *V. cholerae* (*aldA*) is also co-ordinately expressed with CT but AldA has not been shown to be a pathogenicity determinant (Parsot and Mekalanos, 1991).

The regulator of these genes, ToxR, is a membrane-localized transcription activator identified by virtue of its activation of a *ctx–lacZ* gene fusion in *Escherichia coli* (Miller and Mekalanos, 1984, Miller *et al*., 1987). Strains of *V. cholerae* with *toxR* deletion or insertion mutations express undetectable levels of the factors described above (Peterson and Mekalanos, 1988; Miller and Mekalanos, 1988; Herrington *et al*., 1988). Such strains produce the OmpT outer membrane protein and lack expression of OmpU. The absence of virulence-factor production in *toxR* mutant *V. cholerae* is largely due to an inability to express a ToxR-regulated transcription factor called ToxT (see below), leading to a cascade model for virulence gene regulation in *V. cholerae* (DiRita *et al*., 1991; DiRita, 1992). When *toxT* is introduced into *toxR* mutant *V. cholerae* under the control of the *tet* promoter or the *tac* promoter, CT and TCP are expressed constitutively or in the presence of IPTG, respectively (DiRita *et al*., 1991; DiRita *et al*., 1996). Unlike the other ToxR-regulated gene products, OmpU is not restored under these conditions and the cells maintain production of OmpT (DiRita *et al*., 1991).

ToxR shares extensive similarity in its amino terminus with the DNA-binding domain of the OmpR subclass of response regulators in bacteria (Miller *et al*., 1987; Ottemann *et al*., 1992), and mutations in the OmpR-homologous residues abolish the ability of ToxR to bind

Received 3 June, 1996; revised 29 October, 1996; accepted 1 November, 1996. †Present address: Washington University School of Medicine, St. Louis, Missouri 63156, USA. *For correspondence. E-mail vdirita@umich.edu; Tel. (313) 936 3804; Fax (313) 936 3235.

promoter DNA (Ottemann *et al*., 1992; Higgins and DiRita, 1996). The carboxyl terminus of ToxR is in the periplasm (Miller *et al*., 1987), where it interacts with another regulatory protein, called ToxS (Miller *et al*., 1989; DiRita and Mekalanos, 1991). While the nature of this interaction has not been clarified, the current data suggest that it is required for ToxR to assume an active conformation, perhaps dimerization or some other quarternary arrangement (DiRita and Mekalanos, 1991; Dziejman and Mekalanos, 1994; Kolmar *et al*., 1995). Except for homology between the ToxR and OmpR DNA binding domains, ToxR and ToxS share no homology with the two-component family of activators and their interaction may be stoichiometric, rather than catalytic (Miller *et al*., 1989; DiRita and Mekalanos, 1991).

ToxT is an AraC-like protein that activates several genes in the ToxR regulon (Higgins *et al*., 1992; DiRita *et al*., 1991; Ogierman and Manning, 1992). Its carboxyl terminal domain has a helix-turn-helix motif characteristic of this family of activators, and, like other AraC-like proteins, its amino terminus does not share significant similarity to other proteins in the database (Higgins *et al*., 1992). Transcription of *toxT* in *V. cholerae* is, in part, due to a ToxR-dependent promoter present upstream of the initiation of *toxT* transcription. Sequences within this promoter are bound by ToxR in gel-shift experiments (Higgins and DiRita, 1994). Interaction between ToxR and the *toxT* promoter requires the upstream half-site of an inverted repeat element for activation and DNA binding by ToxR (Higgins *et al*., 1992; Higgins and DiRita, 1996). The ToxR-binding site in the *toxT* promoter differs in primary sequence from its binding site in the *ctxAB* promoter, which is characterized by a heptad repeat, TTTTGAT, as well as sequences downstream of the heptad repeat (Miller *et al*., 1987; Pfau and Taylor, 1996). The *toxT* gene is within the *tcp* gene cluster and, in addition to ToxR-dependent transcription activation, *toxT* expression is controlled by transcriptional readthrough from a ToxT-dependent *tcp* promoter (Brown and Taylor, 1995).

A number of observations suggest that virulence-gene expression is not simply a matter of ToxR activating ToxT expression and ToxT activating the genes in the regulon; for instance, both ToxR and ToxT activate the *ctxAB* promoter when tested in *E. coli*. (Miller and Mekalanos, 1984; DiRita *et al*., 1991). The purpose of this dual control is not clear. Also, as described above, OmpU appears to require ToxR, but not ToxT, for its activation, as determined by the observation that ToxT does not restore OmpU production when expressed in a *toxR* mutant background (DiRita *et al*., 1991). Finally, the *acfD* promoter is only weakly activated by ToxT and DNA supercoiling may play a role in its expression (Parsot and Mekalanos, 1992).

To dissect the ToxR/ToxT cascade and to initiate characterization of ToxT functional domains, we constructed mutant derivatives of classical strain O395 and El Tor strain E7946 in which the wild-type *toxT* gene was replaced with an in-frame deletion mutation that removes the coding sequences for the carboxyl terminal helix-turn-helix motif of ToxT. The phenotypes of these mutant strains confirm the existence of ToxT-dependent and ToxT-independent branches in the regulatory cascade and raise questions about the role of ToxR in activating CT gene expression in *V. cholerae*.

Results

Isolation of toxT *deletion derivatives of* V. cholerae *strains O395 and E7946*

Members of the AraC family of transcription activators are characterized by conservation at their carboxyl termini, which includes a helix-turn-helix domain likely to be required for DNA binding (Brunelle and Schleif, 1989). An allele of *toxT* lacking the helix-turn-helix domain ($toxT_{\Delta hth}$), and therefore predicted to be incapable of encoding a protein that can activate transcription, was constructed using a polymerase chain reaction (PCR)-based splicing protocol as described in the *Experimental procedures*.

The wild-type *toxT* alleles in classical strain O395 and in El Tor strain E7946 were both replaced with the mutant allele using *sacB* allelic replacement vector pCVD442 (Donnenberg and Kaper, 1991). The resulting strains were confirmed as carrying the $toxT_{\Delta hth}$ allele by means of PCR analysis using primers flanking the deletion target (data not shown). The E7946$toxT_{\Delta hth}$ strain is called VJ739 and the O395$toxT_{\Delta hth}$ strain is called VJ740.

ToxT is required for TcpA expression, but not for OmpU expression

Based on the fact that ToxT activates the promoter of the *tcp* operon, which encodes the major subunit of the pilus (TcpA) as well as other Tcp products required for pilus assembly (DiRita *et al*., 1991; Brown and Taylor, 1995), we assumed it to be very likely that a *toxT* mutation would result in a severe reduction in detectable TcpA synthesis. Western blot analysis of strains VJ739 and VJ740 using anti-TCP antisera was therefore performed to determine the levels of TcpA produced in the mutant strains. The wild-type El Tor and classical strains E7946 and O395 (respectively) produced detectable TcpA whereas, like *toxR* mutants, *toxT* mutants of both biotypes did not produce TcpA (Fig. 1). Restoration of TcpA production was accomplished by introducing a plasmid that encodes an IPTG-inducible *toxT* gene (Fig. 1) and this was correlated with autoagglutination in liquid culture, a phenotype of TCP-producing *V. cholerae* (data not shown).

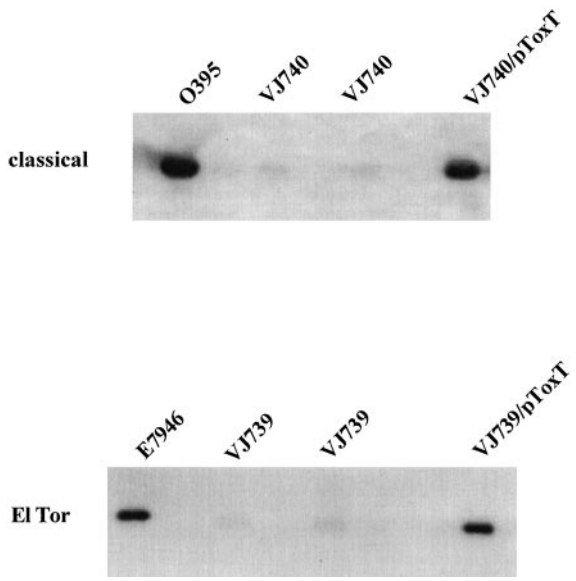

Fig. 1. Immunoblot of TcpA production in wild-type and *toxT* mutant *V. cholerae*. Blots were prepared and probed as described in the *Experimental procedures*. pToxT harbours the *toxT* open reading frame on a low-copy-number plasmid, pMMB66HE, under the control of the IPTG-inducible *tac* promoter. IPTG (1 mM) was used to induce *toxT* transcription in VJ739/pToxT and VJ740/pToxT. O395 and E7946 are wild-type classical and El Tor (respectively) *V. cholerae* strains. Classical strains were grown overnight in LB at 30°C and El Tor strains were grown in AKI medium at 37°C (Iwanaga *et al*., 1986).

While cloned *toxT* restores production of CT and the TCP in *toxR* mutant *V. cholerae*, it does not restore OmpU production, strongly suggesting that ToxT plays no role in *ompU* expression. SDS–PAGE analysis of proteins produced by *toxT* mutant strains VJ739 and VJ740 confirmed this. As shown in Fig. 2, the characteristic shift observed in *toxR* mutants in which OmpU is replaced by OmpT in the outer membrane was not observed in the *toxT* mutant background, and the mutants produced wild-type levels of OmpU.

toxT *mutant* V. cholerae *does not produce CT*

When tested in *E. coli*, both ToxR and ToxT can activate

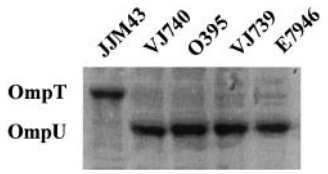

Fig. 2. Outer membrane proteins of wild-type, *toxR* and *toxT* mutant *V. cholerae*. Total proteins were subjected to electrophoresis through 10% SDS–PAGE and stained with Coomassie brilliant blue. Strain JJM43 is a *toxR* mutant derivative of classical strain O395. Cultures were grown overnight at 30°C in LB.

expression of a *ctx–lacZ* gene fusion (DiRita *et al*., 1991; Miller and Mekalanos, 1984). This led us to expect that toxin expression would be unaffected in the *toxT* mutant strains by virtue of *ctx* transcription activation by ToxR. Contrary to our expectations, a GM_1-ELISA assay of culture supernatants revealed that the *toxT* mutants produced undetectable levels of CT (Table 1). As with TcpA, expression of CT could be restored in the mutants by cloned *toxT* (Table 1).

One explanation for our inability to detect CT in the supernatants of the *toxT* mutants is that they may have a defect in secretion of the toxin, rather than in transcription of the genes. The pathway for CT secretion in *V. cholerae* includes disulphide bond formation catalysed by the TcpG(DsbA) protein (Yu *et al*., 1992; Peek and Taylor, 1992), originally identified as a ToxR-regulated gene product (Peterson and Mekalanos, 1988; Peek and Taylor, 1992). A strain deficient for TcpG(DsbA) activity cannot

Table 1. Toxin production in *V. cholerae* wild-type and *toxT* mutants.

Strain	Plasmid	Toxin production (ng/ml/OD_{600})
O395 (classical)[a]	–	295
E7946 (El Tor)[b]	–	768
VJ740 (O395$toxT_{\Delta hth}$)	–	<5
VJ739 (E7946$toxT_{\Delta hth}$)	–	<5
VJ740	pToxT[c]	114
VJ739	pToxT	827
O395	$pToxT_{\Delta hth}$	227
VJ740	$pToxT_{\Delta hth}$	<5
VJ740	pMMB68[d]	254
VJ739	pMMB68	238

a. Supernatants from classical strains were assayed after growth in LB overnight at 30°C.
b. Supernatants from El Tor strains were assayed after overnight growth in AKI conditions.
c. Plasmid experiments included IPTG at 1 mM for induction of genes under *tac* promoter control.
d. pMMB68 encodes the B subunit of the *E. coli* heat-labile toxin (LT-B).

secrete the pentameric B subunit, which is the component of CT that is detected in the GM_1-ELISA assay (Yu *et al*., 1992). We hypothesized that if *tcpG* transcription were under ToxT control, then the mutants might be deficient for CT in the culture supernatants, which is what we use for the GM_1-ELISA.

To test whether toxin secretion was affected in the mutants, we analysed their ability to secrete the B subunit of heat-labile toxin (LT) of *E. coli*, which is identical in structure to CT and which also requires TcpG(DsbA) to be secreted from *V. cholerae* (Yu *et al*., 1992). We introduced pMMB68, which expresses the gene encoding the GM_1-binding subunit of LT (LT-B) from the IPTG-inducible *tac* promoter, into *toxT* mutant strains VJ739 and VJ740. Supernatants from cultures grown with IPTG induction

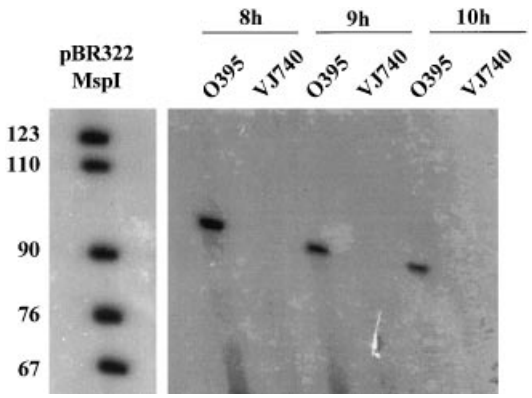

Fig. 3. Primer extension of *ctxAB* transcript in the wild type and the *toxT* mutant classical *V. cholerae* strain O395. See the *Experimental procedures* for details. The bands on the left are those of *Msp*I-digested pBR322 filled in with Klenow fragment in the presence of [α^{32}P]-dCTP.

were assayed by GM_1-ELISA. As shown in Table 1, high levels of LT-B were detected in supernatants from the induced cultures of VJ739(pMMB68) and VJ740(pMMB68) but not from those of the mutants without the plasmids. This result rules out the possibility that our inability to detect CT from supernatants of the mutant *V. cholerae* is due to an effect of the mutation on toxin secretion, and suggests that *ctxAB* transcription *per se* is altered in the *toxT* mutants.

To assess transcription of the *ctxAB* locus in the mutants, we performed primer extension analysis of mRNA from cultures grown under conditions in which the ToxR regulon is active in the wild type. *ctxAB* mRNA levels were assessed from cultures of wild-type (O395) and *toxT* mutant (VJ740) classical *V. cholerae* at 8, 9, and 10 h after overnight dilution of cultures (1:50) in fresh Luria–Bertani (LB) medium, time-points at which we have observed maximal *ctxAB* transcription in the wild type (G. A. Champion and V. J. DiRita, unpublished). As can be seen in Fig. 3, a primer specific for the *ctxA* gene generated a primer extension product from RNA of wild type O395 having a size of ≈105 nucleotides, which is the expected size of a product from this primer based on the previously published *ctxAB* transcription start site (Mekalanos *et al*., 1983). This product was not observed when RNA from the O395 *toxT* mutant derivative VJ740 was used in the primer extension experiment. Agarose-gel and spectrophotometric analyses of the same RNA preparations as those used in the primer extension experiment showed that equivalent amounts of RNA were analysed in each primer extension shown in Fig. 3 (data not shown). We interpret this result as suggesting that *ctxAB* transcription is strongly reduced in the *toxT* mutant background.

Because the lack of toxin production by the *toxT* mutants was unpredicted, we performed immunoblot analysis with anti-ToxR serum to ensure that introduction of the *toxT* deletion alleles did not inadvertently result in a loss of ToxR production. From the immunoblot in Fig. 4, it is clear that the $toxT_{\Delta hth}$ mutation does not appreciably alter ToxR production in *V. cholerae*.

ctx *transcription initiates at the same position with both ToxR and ToxT*

The data presented above suggest that expression of *ctxAB* in *V. cholerae* relies on ToxT, but not on ToxR. Given that ToxR can activate *ctx–lacZ* in *E. coli*, we considered the possibility that this activation may be initiating at a promoter not functional in *V. cholerae*. To test this possibility, we used primer extension to compare the transcription start site of *ctx–lacZ* activated by either ToxR or ToxT in *E. coli* with that of *ctxAB* in wild-type *V. cholerae*.

High-resolution gel electrophoresis of the primer extension products showed that transcription of the *ctx–lacZ* gene fusion appears to initiate at the same position with each activator in *E. coli* (Fig. 5). The initation site with either activator is the same as that observed in wild-type *V. cholerae* strain O395. These results are consistent with the hypothesis that ToxR activates *ctx–lacZ* in *E. coli* from the same promoter as that used in *V. cholerae*.

ToxR and ToxT do not co-operate for ctx *transcription activation*

The $toxT_{\Delta hth}$ mutant strains may not produce CT because the mutant allele might encode a protein that can interfere with the ability of ToxR to activate the *ctx* promoter. This might be expected, for example, if ToxR and ToxT functioned co-operatively to activate the *ctx* promoter, because the mutant ToxT protein might interact aberrantly with ToxR and affect *ctx* transcription. Two predictions arise from the hypothesis that ToxR and ToxT act co-operatively on the *ctxAB* promoter. The first is that expression of the

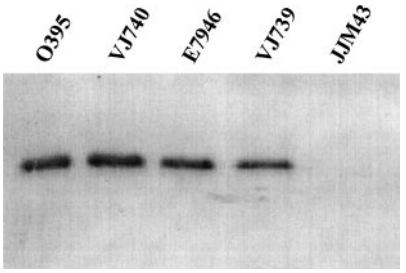

Fig. 4. Immunoblot of ToxR production in *V. cholerae*. Strain JJM43 is a *toxR* mutant derivative of classical strain O395. E7946 is a wild-type El Tor strain. VJ739 and VJ740 are *toxT* mutant derivatives of E7946 and O395, respectively. Cultures were grown overnight at 30°C in LB.

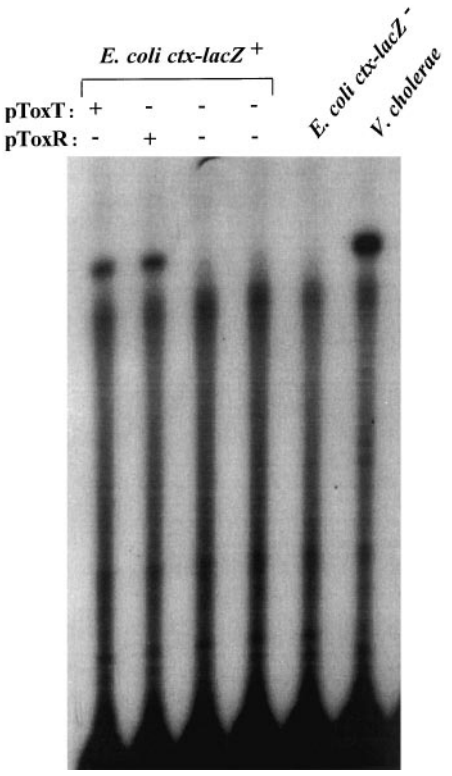

Fig. 5. Primer extension of *ctx–lacZ* mRNA in *E. coli*. See the *Experimental procedures* for details. pToxT is IPTG-inducible pMT5 (DiRita *et al*., 1996) and IPTG was added at 1 mM during cell growth. pToxR is pVJ21, a *toxRS*-containing plasmid previously described by Miller *et al*. (1989). The two lanes lacking pToxR or pToxT represent *E. coli* cells harbouring the cloning vectors for pMT5 and pVJ21. *E. coli ctx–lacZ*$^-$ is strain AAEC189 (Higgins and DiRita, 1994). *V. cholerae* is wild-type classical strain O395. *E. coli* cultures represent 2 h outgrowths of overnight cultures diluted 1:50 in LB with appropriate antibiotics. RNA from the *V. cholerae* culture was isolated after back-dilution (1:50) from overnight into LB and growth for 8 h at 30°C in LB.

mutant allele (lacking the helix-turn-helix coding region) in an otherwise wild-type cell may result in the same phenotype observed in the mutant strains, i.e. the *toxT*$_{\Delta hth}$ mutant allele may show a dominant-negative phenotype in wild-type *V. cholerae*. The second is that we might be able to detect a synergistic effect on *ctx* expression in *E. coli* when ToxR and ToxT are co-expressed as opposed to when they are expressed singly.

The first prediction, i.e. that the *toxT*$_{\Delta hth}$ allele has a dominant-negative phenotype in wild-type *V. cholerae*, was tested by analysing toxin expression in wild-type strain O395 expressing the mutant allele from a plasmid. This strain produces toxin at levels equivalent to the wild-type strain without the mutant allele, ruling out a dominant-negative mutant phenotype (Table 1). The second prediction, i.e. that synergy between ToxR and ToxT for *ctx* activation might be detectable, was tested in *E. coli* strain VM2, which encodes a chromosomal *ctx–lacZ* gene fusion (Miller and Mekalanos, 1984). We assayed β-galactosidase when ToxR alone, ToxT alone, or both were expressed in VM2. Elevated levels of *ctx–lacZ* expression were not observed with both activators, relative to levels of either one separately (Table 2). The weakness of this experiment is that the activators are probably being expressed in *E. coli* at levels different from those in *V. cholerae*, but the simple conclusion we draw from this experiment is that there is no synergy between ToxR and ToxT. We also tested the *toxT*$_{\Delta hth}$ allele for its effect on ToxR activation of *ctx–lacZ*; consistent with the data from *V. cholerae*, there was no difference in ToxR-dependent *ctx* activation.

Taken together, the results presented above are not consistent with the hypothesis that ToxR and ToxT work together for *ctx* activation; they also suggest that the *toxT*$_{\Delta hth}$ allele is a null allele.

The toxT$_{\Delta hth}$ *mutants of* V. cholerae *are attenuated for mouse virulence*

Given that the *toxT* mutant strains produce no detectable TcpA or CT in the laboratory, we considered it unlikely that they would be virulent. However, because ToxR can activate *ctxAB* in *E. coli* in the absence of ToxT, we considered it a formal possibility that ToxR might activate *ctxAB* expression in *V. cholerae* during *in vivo* growth. We therefore tested the virulence of the *toxT* mutant strains in the infant-mouse model of cholera infection.

The data in Table 3 show the results of high-dose infections in three- to five-day-old infant mice. The *toxT* mutations have a severe effect on the pathogenicity of both classical and El Tor strains. Whereas 1.5×10^{10} wild-type classical *V. cholerae* caused death in all eight animals inoculated with it within 24 h, 7×10^{9} *toxT*$_{\Delta hth}$ mutant

Table 2. Expression of *ctx–lacZ* in *E. coli*.

Plasmids in *E. coli* strain VM2 (*ctx–lacZ*)	Miller units of β-galactosidase
pToxT + pACYC184 (+IPTG)	239.7
pToxT + pACYC184 (−IPTG)	38.0
pToxR + pMMB66HE	272.1
pToxR + pToxT (+IPTG)	209.2
pToxR + pToxT (−IPTG)	276.4
pToxT$_{\Delta hth}$ + pToxR (+IPTG)	252.8
pToxT$_{\Delta hth}$ + pACYC184 (+IPTG)	21.2

pToxT, pToxT$_{\Delta hth}$: *toxT* or *toxT*$_{\Delta hth}$ cloned in pMMB66HE; expressed under *tac* promoter control (IPTG-inducible), pToxR, *toxRS* cloned in pACYC184 (expressed under *tet* promoter control (constitutive)).

Table 3. Infant-mouse lethality of wild-type and *toxT* mutant *V. cholerae*.

Strain	Number of Mice Dead At: inoculum	24 hours	36 hours
O395 (wild type, classical)	1.5×10^{10}	8/8	–
VJ740 (O395:$toxT_{\Delta hth}$)	7×10^{9}	0/7	2/7
E7946 (wild type, El Tor)	3×10^{9}	3/8	8/8
VJ739 (E7946:$toxT_{\Delta hth}$)	2.5×10^{9}	0/12	3/12
JJM43 (Δ*toxR* Δ*ctxA* classical)	2.5×10^{9}	1/8	3/8

O395 caused no deaths (out of seven animals inoculated) in 24 h and caused death in only two out of seven by 36 h. Infection with 3×10^{9} cells of the El Tor strain E7946 led to death in three out of eight animals by 24 h and death in all of the animals by 36 h. In contrast, infection with 2.5×10^{9} $toxT_{\Delta hth}$ mutant E7946 resulted in no deaths by 24 h (12 animals tested) and three deaths out of 12 animals by 48 h. The rates of death of the $toxT_{\Delta hth}$ strains are equivalent to that observed with the *toxR ctxA* mutant, JJM43; inoculation with 2.5×10^{9} cells of this strain resulted in death in one animal out of eight at 24 h and three out of eight by 36 h. Death at this later time-point may be due to trauma from the inoculation or perhaps starvation (as three out of eight infant mice inoculated with sterile LB were also dead by 36 h, whereas none of the LB-treated animals was dead at 24 h).

Discussion

Work on the ToxR regulatory system to date has led to a cascade model in which co-ordinate control of virulence gene expression by ToxR is largely, but not solely, the result of its control over ToxT expression (DiRita *et al*., 1991; DiRita, 1992). While strains of *V. cholerae* deficient for ToxR production lack expression of CT, the TCP, and other gene products of the ToxR regulon (Peterson and Mekalanos, 1988), and are avirulent in humans (Herrington *et al*., 1988), expression of CT and the TCP is restored in the $toxR^{-}$ background if *toxT* is expressed from a promoter not regulated by ToxR (DiRita *et al*., 1991; DiRita *et al*., 1996). Unlike toxin and the TCP, however, expression of the ToxR-regulated outer membrane protein OmpU was not restored by overexpression of *toxT* in a *toxR* mutant background (DiRita *et al*., 1991). Because it is clear that the *ctxAB* promoter can be activated to high levels in *E. coli* by supplying either ToxR or ToxT (DiRita *et al*., 1991; Miller and Mekalanos, 1984), the cascade model until now has held that both activators play direct roles in activating *ctxAB* transcription in *V. cholerae* (DiRita, 1992), although the rationale for this dual control has not been clear. Specific predictions from the cascade model were tested in this report by analysing the phenotypes of mutant *V. cholerae* strains lacking a functional ToxT protein by virtue of deletion of sequences encoding the AraC-like putative DNA binding domain.

The data presented here separate the ToxR cascade into ToxT-dependent and ToxT-independent branches (Fig. 6). Based on these data and data from previously published work, the ToxT-dependent branch includes *ctxAB*, *tcp*, at least one of the *acf* genes, *acfA* (Parsot and Mekalanos, 1992), and *aldA*, the gene encoding aldehyde dehydrogenase (DiRita *et al*., 1991; Parsot and Mekalanos, 1991). The ToxT-independent branch consists of *ompU*, as its product is detected at wild-type levels in the *toxT* mutant background (this work) and the *toxT* mutant strain VJ740 supports a wild-type level of expression of an *ompU–lacZ* transcriptional fusion (J. A. Crawford, C. Bailey, J. B. Kaper and V. J. DiRita, unpublished). We infer that ToxR is directly responsible for activation of the *ompU* gene or that it controls an *ompU* activator.

That OmpU is in a separate branch of the ToxR cascade may partly explain why signals that affect expression of toxin and TcpA have less effect on OmpU production (Miller and Mekalanos, 1988). For example, cultures grown in media with a starting pH of 6.5 express ≈100-fold more CT and TcpA than those grown in media with a starting pH of 8.0, but OmpU expression is not detectably different at either pH (Miller and Mekalanos, 1988). We hypothesize that signals modulating the ToxR system have a greater effect on the ToxT-dependent branch than on the ToxT-independent branch. This hypothesis may be explained by the possibility that *toxT* transcription is more sensitive to such signals than *ompU* transcription, or that the magnitude of ToxR-dependent expression of the *ompU* promoter is significantly greater than that of the *toxT* promoter, so that signals impinging on ToxR function would have a relatively more drastic effect on the steady state levels of expression of the ToxT-dependent branch. The latter

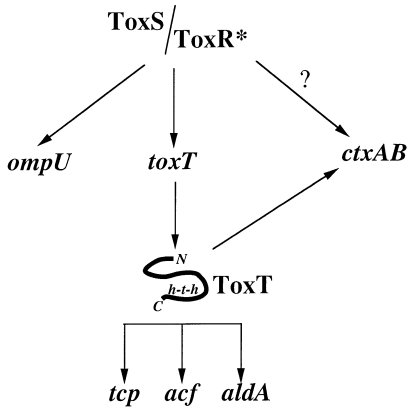

Fig. 6. Model for the ToxR regulatory cascade in *V. cholerae*. ToxR* represents the form of ToxR competent for transcription activation after interaction with ToxS. See the text for details.

possibility is supported by the observation that *ompU* transcription is activated 40-fold by ToxR in *V. cholerae* whereas *toxT* transcription is activated only ≈10-fold (Higgins and DiRita, 1994; J. A. Crawford, C. Bailey, J. B. Kaper, and V. J. DiRita, unpublished).

OmpU dependence on ToxR and ToxS, but not ToxT, supports a model in which ToxR and ToxS may have evolved primarily to control outer membrane protein production in an ancestor of *V. cholerae* that lacked the *tcp–acf* gene cluster and perhaps was not lysogenized with the filamentous *ctxAB*-encoding phage CTXΦ. Once the *tcp–acf* gene cluster had been introduced into the ancestral background, it may have come under ToxR control by virtue of the linked regulatory gene, *toxT*, evolving towards transcriptional control by ToxR. That the CTXΦ phage requires *tcp* expression to provide its receptor (Waldor and Mekalanos, 1996) might provide a rationale as to how toxin synthesis, a phage-encoded function, came to be regulated by the ToxR/ToxT system: the host environment that selected for *tcp* expression and thus phage transmission (Waldor and Mekalanos, 1996) might be most efficiently colonized if toxin were also expressed. This model for convergent evolution of ToxR-dependent activation does not specifically predict what the selection pressures were that resulted in toxin and pilus production becoming co-ordinate with that of OmpU, but evidence from other systems supports the idea that ToxR might have evolved originally to control outer membrane protein production in *V. cholerae*. Investigators studying *Vibrio fischeri* and *Photobacterium* sp. strain SS9, two marine microbes, have shown that mutations in genes that encode ToxR homologues in these species result in alterations in outer membrane protein production (K. A. Reich, personal communication, and D. H. Bartlett, personal communication).

The role of OmpU in *V. cholerae* virulence has not been established. Antisera directed against OmpU blocks adhesion of *V. cholerae* to HEp-2 cells *in vitro* and protects infant mice from lethal challenge with *V. cholerae* (Sperandio *et al*., 1995). The amino terminus of OmpU shares a region similar to adhesins from a diverse array of Gram-negative organisms (Sperandio *et al*., 1996). Taken together, these results suggest that OmpU is an adhesin essential for wild-type colonization, but strains with defined mutations in the gene have yet to be constructed and characterized.

The lack of CT production by *toxT* mutant strains, in which ToxR should still function, was not predicted because ToxR can activate *ctx* transcription in *E. coli*. For the following reasons we consider it unlikely that we inadvertently acquired a *toxR* mutation in these strains. First, the level of ToxR detected by immunoblot analysis in the mutants was comparable to that of the wild type and the antigen detected in the mutants is of the same relative mobility as that in the wild type, thus ruling out a gross defect in ToxR production. Second, OmpU expression is completely unaffected in the mutants. Mutations in *toxR* or *toxS* typically result in a phenotype in which cells express reduced levels of OmpU and increased levels of OmpT. We think it improbable that we acquired a spontaneous *toxR* mutation that results in an $OmpU^+$ Ctx^- phenotype in two independently isolated *toxT* mutant strains. Third, CT production was not restored to significant levels in the *toxT* mutants by introduction of a plasmid encoding functional ToxR and ToxS (data not shown). Notwithstanding these arguments, it remains a formal possibility that we have acquired, in two independently derived *toxT* mutant isolates, alleles of *toxR* that encode a protein deficient for *ctx* expression, but not for *ompU* expression, and which are dominant over the wild type. This possibility is being tested by cloning the *toxRS* operons from the *toxT* mutants and determining whether they encode proteins that can activate *ctx*::*lacZ* in *E. coli*.

The question of transcriptional control of CT in *V. cholerae* requires more work for complete clarification. It is highly unlikely that *ctx–lacZ* activation by ToxR in *E. coli* is an artifact because transcription initiation occurs at the same position as it does in *V. cholerae* (Fig. 4) and is associated with DNA binding by ToxR to specific sequences within the *ctxAB* promoter (Miller *et al*., 1987; Pfau and Taylor, 1996). The lack of CT expression in $toxR^+$ $toxT^-$ cells may provide a basis for an understanding of why there are two activators that can work independently in this system. Perhaps a factor required for ToxR to activate *ctxAB* in *V. cholerae* is ToxT dependent, but a functionally interchangeable factor is present in *E. coli* in the absence of ToxT. Alternatively, perhaps the conditions under which ToxR can activate *ctxAB* transcription in *V. cholerae* are not met by the way in which cultures are grown in the laboratory (though this would mean that overexpression of ToxR in *E. coli* grown in the same way would have to meet these conditions). If this were so, then there might be growth conditions in which the *toxT* mutant of *V. cholerae* is able to express CT. A model derived from this possibility is that at some stage of the infection process ToxR is required for activating both *ctxAB* and *toxT*. After that, ToxR is no longer required because ToxT takes over expression of *ctxAB* and of its own transcription, by virtue of readthrough transcription of *toxT* in the *tcp* gene cluster (Brown and Taylor, 1995). This possibility is currently under investigation.

Experimental procedures

Bacterial strains and plasmids

E. coli and *V. cholerae* strains were maintained at −70°C in LB medium containing 25% glycerol. *V. cholerae* strains were grown at 25–30°C in LB medium or in AKI conditions at

37°C (Iwanaga *et al*., 1986). Antibiotics were used at the following concentrations: ampIllicin, 100 μg ml^{-1}; tetracycline, 12.5 μg ml^{-1}; kanamycin, 30 μg ml^{-1}; and streptomycin, 100 μg ml^{-1}. Plasmids were introduced into *E. coli* strains by transformation and into *V. cholerae* strains through electroporation or through triparental mating with *E. coli* strain MM294(pRK2013) as a donor of mobilization functions.

DNA manipulation

Plasmid DNA was purified with Qiagen columns (QIAGEN, Inc.). Cloning was performed using standard protocols (Sambrook *et al*., 1989). PCR was performed with Vent^{R} DNA polymerase (New England Biolabs) using manufacturer's protocols. A PCR splicing protocol using primers within and surrounding *toxT* was performed to create an in-frame deletion that removes the DNA encoding the helix-turn-helix domain of ToxT using pDH8 DNA (Higgins *et al*., 1992) as a template. The procedure requires two PCR reactions, as follows. A primer (5′-CGTAGTACTGAGCAGCGCCAGAGCTC-TCTCCTCACATT-3′) homologous to the 3′ end of the *tcpE* gene, which is upstream of *toxT*, and a primer (5′-TGTC-AACATAAATAAATATTCTTCACCGGTTTTCATTAGTG-3′) homologous to the DNA on either side of the helix-turn-helix sequence in the *toxT* open reading frame (Higgins *et al*., 1992), were used for the first reaction to produce PCR I (951 bp). This fragment was annealed to a restriction fragment whose 5′ end was homologous to the 3′ end of PCR I and which proceeded through the downstream *tcpJ* gene. The annealed template was then used in a second PCR reaction with the *tcpE* primer from the first PCR reaction as the upstream primer and a primer homologous to the *tcpJ* gene (5′-GCTGGAGCTCATGCATCTGAAAGTAAGTAAGGATAG-3′) as the downstream primer.

The outermost primers in this splicing protocol contained the site for the restriction enzyme *Sac*I (underlined text in the primer sequences above), which allowed the final PCR product, encoding the $toxT_{\Delta hth}$, to be cloned into the *Sac*I site of the *sacB* positive selection plasmid, pCVD442 (Donnenberg and Kaper, 1991). The resulting plasmid was introduced into *V. cholerae* classical strain O395 or El Tor strain E7946 by conjugation selecting for resistance to plasmid-encoded ampicillin. Loss of ampicillin resistance and exchange of wild-type *toxT* with the $toxT_{\Delta hth}$ allele was accomplished by selecting for resistance to sucrose as described by Donnenberg and Kaper (1991). DNA from isolates that were both sensitive to ampicillin and resistant to sucrose was analysed by PCR using the upstream primer 5′-CGTTCACTAAAATCTTACATTC-3′ and the downstream primer 5′-GTGATACAATCGAAAATAGG-3′. DNA amplified with these primers should give a fragment of 596 nucleotides from DNA of the wild type and a fragment of 506 nucleotides from DNA of the deletion mutant.

RNA analysis

RNA was obtained from cultures of *V. cholerae* and *E. coli* using Tri-Reagent (Molecular Research Centre) as described (Higgins and DiRita, 1994) according to the manufacturer's protocols. When RNA was isolated from *V. cholerae* for *ctx* primer extension, an overnight culture grown in LB at 30°C to late log phase (OD_{600} = >3.0) was subcultured 1:50 into fresh LB media. At one-hour time-points, aliquots of the cultures were removed and poured over crushed ice prior to centrifugation for cell recovery. If cell pellets were not used for RNA treatment using Tri-Reagent immediately they were stored at −20°C until ready for use. Primer extension of *ctx* and *ctx–lacZ* transcripts was performed as described (Higgins and DiRita, 1994) using the *ctxA*-specific primer 5′-GAATCTGCCCGATATAACTTATCATCATTTGCAT-3′.

Protein analysis

Total cell lysates were prepared from *V. cholerae* cells grown to stationary phase overnight and back-diluted 1:100 into LB medium for 3 h. From the cultures, 1 ml was removed and centrifuged. The harvested pellet was resuspended in SDS–PAGE sample buffer and boiled for 5 min. Aliquots of this lysate were subjected to electrophoresis on a 10% polyacrylamide gel with a 5% stacking gel. For Western blots, the gels were blotted onto nitrocellulose and probed separately with anti-TcpA and anti-ToxR rabbit monoclonal antibodies at dilutions of 1:2000. CT was quantified from supernatants of cultures using the GM_1 ganglioside ELISA assay (Svennerholm and Holmgren, 1978).

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