Molecular Microbiology (2000) **38**(1), 67–84

The *Vibrio cholerae* ToxR/TcpP/ToxT virulence cascade: distinct roles for two membrane-localized transcriptional activators on a single promoter

Eric S. Krukonis,[1] Rosa R. Yu[2] and Victor J. DiRita[1,2]*
[1]*Unit for Laboratory Animal Medicine, and*
[2]*Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109-0614, USA.*

Summary

ToxR is required in *Vibrio cholerae* for transcriptional activation of the *toxT* gene, the protein product of which activates numerous genes involved in virulence. Although ToxR cannot activate the *toxT* promoter in *Escherichia coli*, the products of the *tcpPH* operon are shown here to activate the *toxT* promoter, and co-expression with ToxRS enhances activation. An identical pattern was seen in a Δ*tcpP*Δ*toxR* strain of *V. cholerae* when TcpPH or ToxRS was expressed from plasmids. Although overexpression of the TcpP/H proteins in *V. cholerae* partially complemented both a Δ*toxR* strain and a Δ*tcpP*Δ*toxR* double mutant for toxin production and *toxT–lacZ* activation, the presence of ToxR greatly increased their expression. Analysis of a *toxT–lacZ* promoter deletion series demonstrated that TcpP was able to interact functionally with the *toxT* promoter downstream of the ToxR binding site. This was confirmed using electrophoretic mobility shift assays of this *toxT* promoter deletion series and DNase I footprinting analysis, which showed that TcpP interacts with the promoter region from −51 to −32, whereas ToxR protected a region from −100 to −69. In addition, membranes containing endogenous levels of ToxR bound more readily to the *toxT* promoter than did membranes containing only TcpP. Characterization of a number of *tcpP* substitution mutants revealed one derivative (TcpP-H93L) that, when overexpressed, was markedly defective for *toxT* activation, cholera toxin and TcpA (toxin co-regulated pilus) production and DNA binding; however, *toxT* activation by TcpP-H93L was restored in the presence of ToxR, suggesting that ToxR can provide the promoter recognition function for *toxT* activation. Two additional mutant derivatives, TcpP-W68L and TcpP-R86A, failed to activate *toxT* or direct toxin and TcpA production in the presence or absence of ToxR. Both TcpP-W68L and TcpP-R86A, like TcpP-H93L, were defective for DNA binding. Finally, a ToxR mutant derivative, ToxR-G80S, served to separate the different roles of ToxR on different promoters. Although ToxR-G80S was inefficient at activating the *ompU* promoter in *V. cholerae* (*ompU* encodes an outer membrane porin regulated by ToxR), it was fully capable of activating the *toxT* promoter. These data suggest that ToxR is not a direct activator in the *toxT* expression system but, instead, enhances the activity of TcpP, perhaps by recruiting it to the *toxT* promoter under conditions in which expression levels of TcpP are too low for it to activate *toxT* efficiently on its own.

Accepted 14 July, 2000. *For correspondence. E-mail vdirita@umich.edu; Tel. (+1) 734 936 3804; Fax (+1) 734 936 3235.

© 2000 Blackwell Science Ltd

Introduction

Vibrio cholerae is the causative agent of the diarrhoeal disease cholera. Secretion of cholera toxin by the bacterium leads to elevated cAMP levels in the cells lining the host intestinal tract, resulting in voluminous secretion of water and electrolytes. Although cholera toxin is the effector of these changes in host physiology, a number of other factors are known to be critical for establishing a productive infection. These include the regulatory proteins ToxR and ToxT (Miller *et al*., 1987; DiRita *et al*., 1991), the colonization factor toxin co-regulated pilus (TCP, encoded by tcpA; Taylor *et al*., 1987), several accessory colonization factors (AcfA–D; Peterson and Mekalanos, 1988) and the recently identified response regulator homologue *varA* (Wong *et al*., 1998). ToxR and ToxT operate in a cascade-like fashion in which ToxR activates the *toxT* promoter, and ToxT acts downstream to regulate various promoters, including *ctxA*, *tcpA*, *tcpC*, *tcpI* and *acfA* (DiRita *et al*., 1991). Mutations in *toxR* result in loss of production of all these factors as well as the ToxT-independent product OmpU (Miller and Mekalanos, 1988), an outer membrane porin (Chakrabarti *et al*., 1996), whereas mutations in *toxT* prevent production of all these factors with the exception of OmpU (Champion *et al*., 1997). Furthermore, heterologous expression of ToxT can bypass the effects of a *toxR* mutation, except with respect to OmpU (Champion *et al*., 1997).

ToxR appears to have gained control of virulence gene

expression via ToxT after an evolutionary event in which the *tcp–acf* gene cluster (the Vibrio pathogenicity island, VPI), which includes *toxT*, was delivered to *V. cholerae* by horizontal transfer via the newly described VPI phage (Kovach *et al*., 1996; Karaolis *et al*., 1998; 1999). Similarly, the cholera toxin locus is also on a mobile genetic element, the CTX phage (Waldor and Mekalanos, 1996). Thus, both the major virulence determinant of *V. cholerae*, cholera toxin, and its regulator, ToxT, were obtained by independent horizontal transfer events.

Recently, a second level of regulation was shown to impinge on *toxT* expression. Mutations in the *tcpPH* locus lying upstream of the TCP biosynthetic operon led to loss of *toxT* transcription (Carroll *et al*., 1997; Häse and Mekalanos, 1998; Yu and DiRita, 1999). Additionally, overexpression of the TcpP protein activates *toxT* transcription, as measured by both expression of a *toxT–lacZ* gene fusion and primer extension analysis in *V. cholerae* (Häse and Mekalanos, 1998; Yu and DiRita, 1999). Thus, transcription of the major regulator of virulence factor transcription, *toxT*, is dependent on the activities of two transcription factors, ToxR and TcpP.

ToxR and TcpP are homologous to the OmpR family of transcriptional activators. Unlike many OmpR family activators, both proteins contain DNA-binding motifs in their N-termini rather than in their C-termini (Miller *et al*., 1987; Ogierman *et al*., 1996), and both are predicted to reside within the inner membrane of the bacterium with substantial periplasmic domains: 96 (ToxR) and 52–60 (depending on the location of the putative transmembrane domain of TcpP) amino acids (Miller *et al*., 1987; DiRita and Mekalanos, 1991; Ogierman *et al*., 1996). In addition, both *toxR* and *tcpP* are encoded in polycistronic operons with downstream genes (*toxS* and *tcpH* respectively) encoding effector proteins that enhance the activity of their cognate partners (Carroll *et al*., 1997; Yu and DiRita, 1999; E. S. Krukonis, V. J. DiRita and B. Hammer unpublished observations).

Transcription of *tcpPH* has been shown to be regulated by both temperature and pH (Thomas *et al*., 1995; Carroll *et al*., 1997), raising the possibility that environmental regulation of the ToxR/TcpP/ToxT cascade in *V. cholerae* manifests itself at the level of TcpP production. Transcription of *tcpPH* has recently been shown to be controlled by two additional factors, AphA and AphB (Kovacikova and Skorupski, 1999; Skorupski and Taylor, 1999).

In this study, we undertook a mechanistic analysis of *toxT* transcriptional regulation by ToxR and TcpP. We show that, as predicted from previous studies demonstrating the inability of ToxR alone to activate *toxT* (Higgins and DiRita, 1994), TcpP functions with ToxR for *toxT* activation, consistent with what has been reported recently by others (Häse and Mekalanos, 1998; Murley *et al*., 1999). We expand this observation to show that TcpP binds to the *toxT* promoter at a position downstream of the ToxR binding site, closer to the basal elements of the promoter. We present a model for the interaction of ToxR and TcpP with the *toxT* promoter, in which ToxR facilitates the ability of TcpP to interact with RNA polymerase, leading to transcriptional activation of *toxT* and subsequent coordinate activation of virulence gene expression.

Results

TcpP can activate a toxT–lacZ *fusion in* E. coli*, but not* ompU–lacZ *or* ctxA–lacZ

Previous studies in *Escherichia coli* demonstrated that ToxR alone is insufficient to activate the *toxT* promoter (Higgins and DiRita, 1994). Furthermore, recent evidence from *V. cholerae* showed TcpP to be required for the expression of *toxT* (Carroll *et al*., 1997; Häse and Mekalanos, 1998). Thus, we sought to determine whether TcpP could activate *toxT* transcription in *E. coli* and whether ToxR played a role in *toxT* activation.

An *E. coli* strain harbouring a chromosomally located *toxT–lacZ* fusion (DH92; Higgins and DiRita, 1994) was transformed with an IPTG-inducible plasmid harbouring the *tcpPH* locus of classical *V. cholerae* strain O395 and compared with the strain carrying the vector alone (pMMB66EH; Fürste *et al*., 1986). TcpPH was able to activate the *toxT* promoter strongly, inducing activation approximately eightfold, whereas plasmid alone had no effect (Fig. 1A; pACYC + TcpPH). A similar induction was seen when the *tcpPH* locus from El Tor strain E7946 was used (data not shown). In order to investigate the effect of ToxR on this TcpPH-dependent activation, we introduced ToxRS into the *E. coli* reporter strain in the presence or absence of TcpPH. As shown previously, ToxRS alone (ToxRS + pMMB66EH) repressed activation in this background twofold (Higgins and DiRita, 1994), reflecting the fact that ToxR binds to the *toxT* promoter but cannot activate transcription, and actually inhibits basal transcription (Fig. 1A; Higgins and DiRita, 1994). When ToxRS and TcpPH were co-expressed, activation mediated by TcpPH was consistently enhanced (from 1250 to 1800 units in Fig. 1A). Activation resulting from low-level TcpPH expression in the absence of IPTG in conjunction with ToxRS was also slightly elevated over that observed with uninduced TcpPH alone (Fig. 1A; ToxRS + TcpPH −IPTG). Thus, ToxR and TcpP in combination give maximal activation of the *toxT* promoter.

Two other ToxR-regulated promoters, *ompU* and *ctxA*, were examined for TcpP-dependent activation by expressing TcpPH, ToxRS or both pairs of proteins in an *E. coli* strain harbouring *ompU–lacZ* (AC174; Crawford *et al*., 1998) or *ctxA–lacZ* (VJ787; A. Bock and V. J. DiRita, unpublished) at the same chromosomal location as the

toxT–lacZ fusion described above (Elliott, 1992). ToxRS activated the *ompU–lacZ* fusion to high levels (≈100-fold) as seen previously (Crawford *et al*., 1998), whereas TcpPH failed to activate *ompU* (Fig. 1B). In addition, no enhancement of ToxR-dependent activation of *ompU* was seen in the presence of TcpPH (Fig. 1B). Thus, TcpPH appears to play no role in *ompU* regulation, which could be predicted from the observation that *ompU* is in the ToxT-independent branch of the *V. cholerae* ToxR regulatory cascade (Champion *et al*., 1997).

As the *ctxAB* promoter is also known to be activated by ToxR in *E. coli*, we investigated whether TcpP would have any effect on its activation. Strains expressing ToxRS gave strong (≈30-fold) activation of the *ctxAB* promoter (Fig. 1C). However, expression of TcpPH alone or in combination with ToxRS had no effect on transcription. Taken together, we conclude that functional interaction between ToxR and TcpP for transcriptional activation is restricted to the *toxT* promoter.

Expression of both TcpPH and ToxRS is required for maximal TcpA and toxin production in V. cholerae

Previous studies have shown TcpP is required for *toxT*, *tcpA* and *ctxAB* activation in *V. cholerae* (Carroll *et al*., 1997; Häse and Mekalanos, 1998; Murley *et al*., 1999; Yu and DiRita, 1999). In order to assess the molecular basis for this observation, we constructed a Δ*tcpP* mutant of *V. cholerae* for use in the experiments described below. As expected, the Δ*tcpP* derivative was defective in both TcpA expression and toxin production (Fig. 2, lane 2), whereas OmpU production was unaffected (data not shown). Plasmid complementation with *tcpPH* restored both TcpA and toxin production to greater than wild-type levels, suggesting that TcpP is the limiting factor in this system (Fig. 2, lane 3).

Based on the evidence from *E. coli* shown above, TcpP appears to be sufficient for the activation of *toxT*, albeit not maximal activation. To determine the role of ToxR in

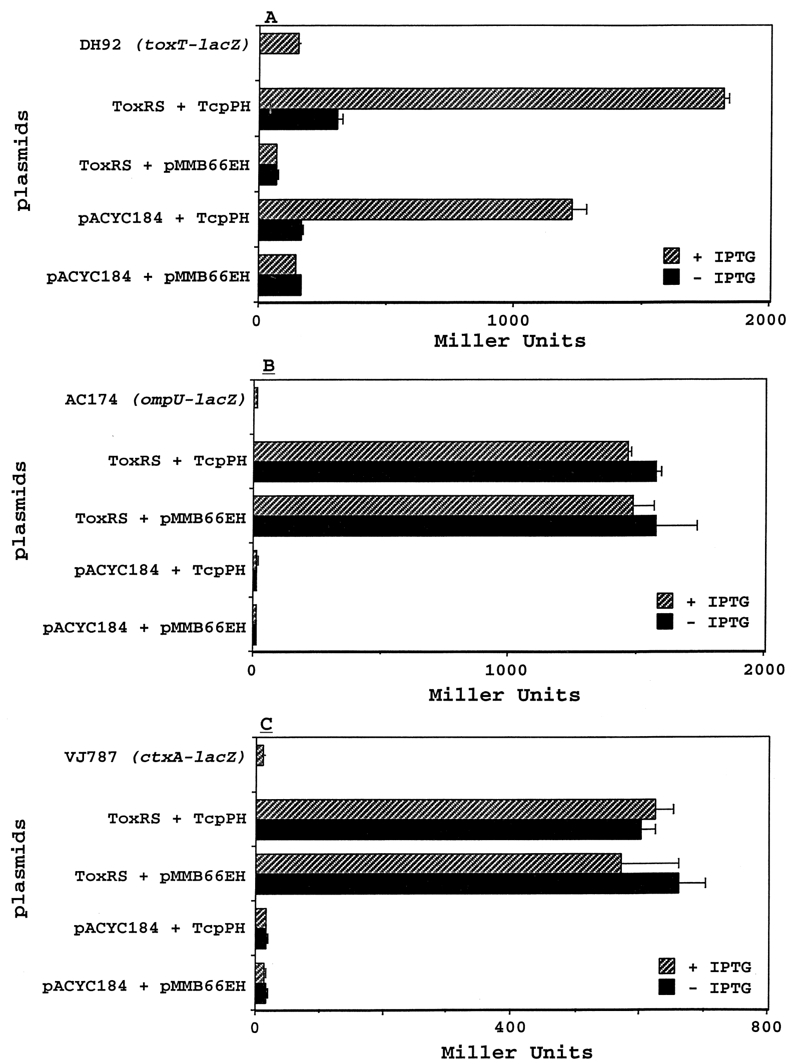


Fig. 1. TcpP-mediated activation of *toxT–lacZ* (A), *ompU–lacZ* (B) and *ctxAB–lacZ* (C) fusions located on the *E. coli* chromosome at the *put* operon locus (Elliott, 1992; Higgins and DiRita, 1994; Crawford *et al*., 1998; A. Bock and V. J. DiRita, unpublished observations). Plasmids encoding TcpPH under the control of an IPTG-inducible promoter or vector alone (pMMB66EH) were co-transformed with a constitutive expression plasmid encoding ToxRS (pVJ21) or an empty vector (pACYC184) into the appropriate *E. coli lacZ* fusion strain. Co-expression of *toxRS* allowed for the analysis of TcpPH–ToxRS synergistic effects.

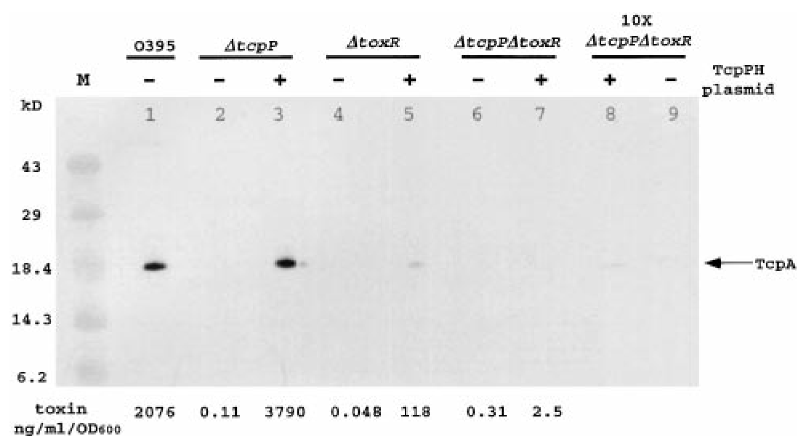


Fig. 2. Effect of ToxR on virulence gene expression. *V. cholerae* mutant strains were grown overnight in LB and 1 mM IPTG at 30°C in the presence of an inducible plasmid harbouring *tcpPH* or empty vector. Cell supernatants were used to measure cholera toxin levels, and cell pellets were used for TcpA Western blotting (*Experimental procedures*). In order to see TcpA in the *tcpPH*-complemented *Δ tcpPΔ toxR* strain, 10-fold more cell extract was loaded (lanes 8 and 9).

enhancing *toxT* expression in its natural background, we constructed *Δ toxR* and *Δ toxRΔ tcpP* strains of *V. cholerae*. A *Δ toxR* mutant (EK307) was partially complemented for TcpA and toxin production by overexpressing TcpPH from a plasmid (Fig. 2; lane 5), although expression levels of both cholera toxin and TcpA were far lower than those in the presence of ToxR (Fig. 2; lanes 1 and 3 versus lane 5). A *Δ toxRΔ tcpP* double mutant (EK459) complemented with TcpPH expressed even less cholera toxin and TcpA than the single *Δ toxR* mutant, presumably reflecting lower overall TcpP levels resulting from the lack of chromosomally encoded TcpP in conjunction with plasmid-expressed TcpP (Fig. 2, lanes 5, 7 and 8). From these data, it is clear that, although TcpP is sufficient for intermediate levels of activation of the *toxT* promoter, ToxR acts to enhance this activation strongly.

TcpP interacts on the toxT *promoter downstream of ToxR*

As ToxR has been shown to interact with a region of the *toxT* promoter upstream of the −73 position, yet is unable to activate this promoter (Higgins and DiRita, 1994; Li *et al*., 2000), we next sought to gain a more mechanistic understanding of the roles of both TcpP and ToxR in *toxT* promoter activation. To determine the *toxT* promoter sequences required for TcpP or ToxR interaction, a series of *toxT–lacZ* promoter deletion constructs was analysed in the *V. cholerae Δ tcpPΔ toxR* mutant for the ability of either TcpP or ToxR expressed from a plasmid to activate transcription. Four previously characterized deletion constructs were analysed, with 5′ end-points at nucleotides −172 (full length), −114, −73 and −46. A −172 derivative truncated to delete the −10 consensus RNA polymerase (RNAP) binding region (Δ-10) was also analysed (Fig. 3A; Higgins and DiRita, 1994). All constructs had nucleotide +45 as a 3′ end-point, except the Δ-10 derivative.

TcpPH activated the −172, −114 and −73 promoter constructs when expressed from a plasmid in the *Δ toxRΔ tcpP* background. Neither the −46 nor Δ-10 construct could be activated by TcpPH (Fig. 3B). ToxR binding to the *toxT* promoter was monitored as repression of basal *toxT–lacZ* activation, because twofold repression of the *toxT* promoter has been documented previously in *E. coli* and is presumed to reflect ToxR binding and repression of a weak cryptic promoter upstream of the *toxT* promoter (Higgins and DiRita, 1994). In the absence of TcpP, overexpressed ToxRS repressed β-galactosidase activity of only the −172 and −114 promoter constructs (Fig. 3C), in agreement with data mentioned above demonstrating that ToxR requires sequences upstream of −73 in the *toxT* promoter to activate its expression in *V. cholerae* (Higgins and DiRita, 1994). Thus, TcpP is able to interact functionally with a *toxT* promoter deleted for the ToxR binding site, suggesting that the TcpP binding site is downstream of that for ToxR.

The *toxT–lacZ* deletion plasmids were next introduced into the *Δ tcpP* and *Δ toxR* single mutant strains to assess the role of ToxR on *toxT* promoter activation in *V. cholerae*. The *Δ toxR* mutant failed to activate any of the five *toxT* promoter constructs, demonstrating that ToxR is required for *toxT* activation at wild-type TcpP expression levels (Fig. 3D). This is in agreement with previous studies using a different *Δ toxR* mutant strain (JJM43; Higgins and DiRita, 1994). Overexpressed TcpP provided from a plasmid was able to restore an intermediate level of *toxT* activation to the *Δ toxR* strain (Fig. 3D).

None of the *toxT–lacZ* deletion derivatives were activated in the *Δ tcpP* mutant background (Fig. 3D). Complementation of the *Δ tcpP* strain with plasmid-encoded *tcpPH* restored activation to high levels (Fig. 3D). Most notably, activation of the full-length *toxT* promoter (−172) and the −114 deletion construct, both of which harbour the ToxR binding site, was to much higher levels in this background compared with the *Δ toxR* and *Δ toxRΔ tcpP* mutant strains. This enhanced activation is probably caused by the action of ToxR in the *Δ tcpP* background, allowing maximal activation of ToxR-recognized promoters

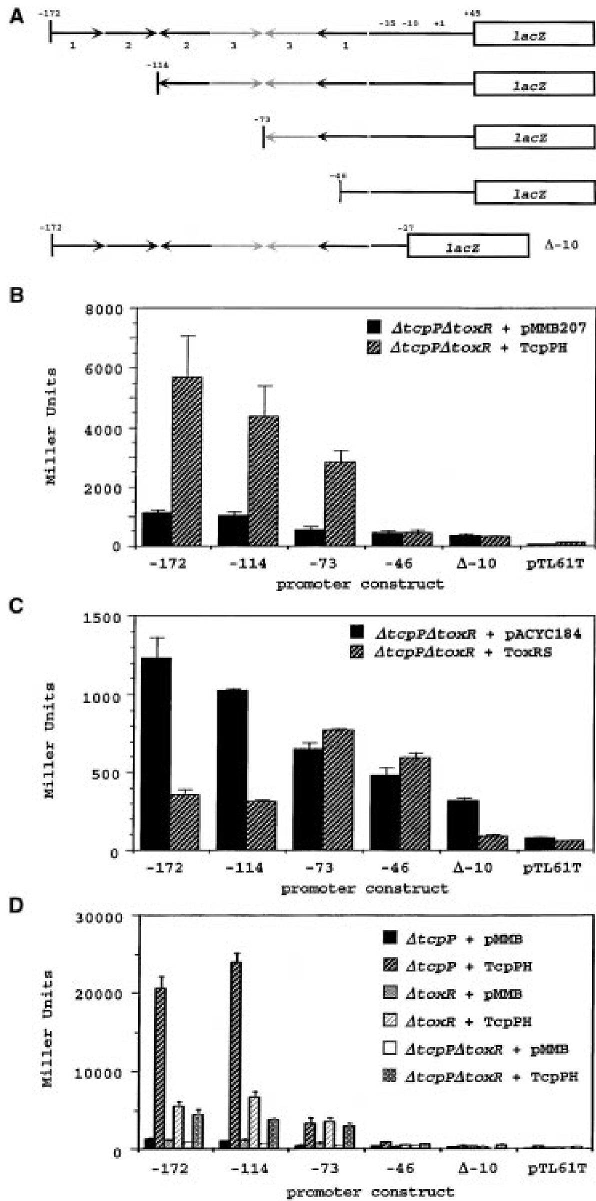


Fig. 3. Activation of various *toxT–lacZ* deletions.
A. Structure of *toxT* promoter deletion constructs (Higgins and DiRita, 1994).
B. TcpPH-directed activation of *toxT–lacZ* in *V. cholerae*. An inducible *tcpPH*-encoding plasmid or vector alone (pMMB207) in conjunction with various *toxT–lacZ* fusion plasmid constructs were introduced into a *V. cholerae* Δ*tcpP*Δ*toxR* strain (EK459), and β-galactosidase activity was measured after a 4 h induction with 1 mM IPTG at 30°C.
C. ToxR-dependent repression of the *toxT* promoter in *V. cholerae*. A constitutive *toxRS*-encoding plasmid or vector alone (pACYC184) in conjunction with various *toxT–lacZ* fusion plasmid constructs was introduced into a *V. cholerae* Δ*tcpP*Δ*toxR* strain (EK459). Cells were diluted 1:100 from an overnight culture, induced for 4 h at 30°C, and β-galactosidase activity was measured.
D. The effect of ToxR on *toxT–lacZ* induction by TcpP. An inducible plasmid encoding *tcpPH* or vector alone (pMMB207) in conjunction with various *toxT–lacZ* fusion plasmid constructs was introduced into *V. cholerae* Δ*tcpP* (RY1), Δ*toxR* (EK307) and Δ*tcpP*Δ*toxR* (EK459) strains. Cells were induced with 1 mM IPTG for 4 h at 30°C, and β-galactosidase activity was measured.

(−172 and −114) by TcpP, and is similar to *toxT–lacZ* expression levels seen in the wild-type strain O395 (data not shown; Higgins and DiRita, 1994).

Together, these data demonstrate that ToxR and TcpP interact with the *toxT* promoter at distinct positions required for activation. ToxR is positioned upstream of −73, whereas TcpP requires a site downstream of −73. This would place TcpP in a position closer to the basal promoter elements and presumably in closer proximity to RNAP than ToxR. Our data also suggest that TcpP is the direct activator in this system and that ToxR provides a supporting role, as evidenced by the facts that TcpP overexpression alleviates ToxR dependence and ToxR alone cannot active *toxT* expression.

TcpP- and ToxR-dependent toxT *promoter interactions*

To assess the DNA-binding properties of TcpP and ToxR on the *toxT* promoter, membranes from four *V. cholerae* derivatives were prepared and analysed by an electrophoretic mobility shift assay (EMSA) using the −172 to +45 *toxT* fragment as a probe. The strains used were O395 (wild type), EK307 *(*Δ*toxR)*, RY1 *(*Δ*tcpP)* and EK459 *(*Δ*tcpP*Δ*toxR)*. Membranes were prepared from cells grown under conditions that induce high levels of toxin and TCP pilus expression (Miller *et al.*, 1987; Miller and Mekalanos, 1988). Whereas membranes lacking both TcpP and ToxR showed negligible binding to the −172 to +45 *toxT* promoter construct, even at the highest concentration of total protein tested (Fig. 4; lane 4), membranes harbouring TcpP showed binding to the *toxT* promoter at this concentration relative to the $ToxR^{-}TcpP^{-}$ control (Fig. 4; lane 8 versus lane 4). In contrast, membranes containing either ToxR alone or ToxR and TcpP bound the *toxT* probe at 10-fold lower concentrations of membrane protein than those with TcpP alone (Fig. 4; lanes 10 and 14), indicating that ToxR binds the *toxT* promoter more efficiently than TcpP under conditions in which virulence gene expression is induced. This could reflect a difference in relative DNA-binding affinities, protein expression levels or both.

TcpP binds downstream of ToxR on the toxT *promoter*

The *toxT–lacZ* expression experiments described above indicate that TcpP can interact functionally with the *toxT* promoter downstream of ToxR. We sought to test this conclusion biochemically by assessing the ability of TcpP and ToxR to bind different regions of the *toxT* promoter. To determine which region of the *toxT* promoter TcpP binds, *V. cholerae* membranes harbouring TcpP, ToxR or neither protein were analysed at 2.5 mg ml^{-1} or 10 mg ml^{-1} membrane protein for their ability to shift

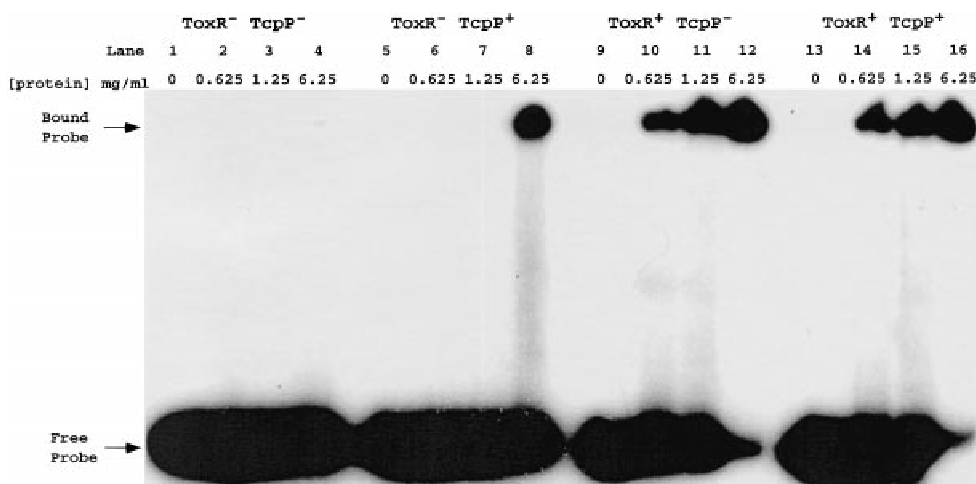


Fig. 4. Binding of full-length *toxT* promoter to *V. cholerae* cellular membranes containing ToxR, TcpP or neither protein. End-labelled probe containing the −172 to +45 region of the *toxT* promoter was incubated with increasing concentrations of membrane for 30 min at 30°C and resolved in a 6% non-denaturing polyacrylamide gel. The ToxR and TcpP proteins were expressed from genes at their normal chromosomal locus.

radioactively labelled DNA fragments corresponding to the various *toxT* promoter deletions described above.

Membranes from a strain deleted for both *tcpP* and *toxR* showed low levels of binding to all probes tested, although background binding to the −172 probe at the higher concentration of membrane was significant (Fig. 5; lanes 3, 12 and 21). This increase in background binding compared with the previous experiment (Fig. 4) may reflect the fact that slightly higher concentrations of membrane were used (10 mg ml^{-1} versus 6.25 mg ml^{-1}) or the inherent variability in the assay from experiment to experiment. Membranes containing TcpP, but not ToxR, bound each promoter construct more readily than the $TcpP^{-}ToxR^{-}$ control, except that in which the −46 to −73 region was deleted (Fig. 5; lanes 5, 14 and 23; −46 to +45 data not shown). Consistent with the binding experiment using a full-length *toxT* promoter (Fig. 4), the $TcpP^{+}ToxR^{-}$ membranes showed weak promoter interaction at 2.5 mg ml^{-1} membrane protein, but bound well at 10 mg ml^{-1} protein. The membrane preparation from a strain expressing only ToxR, on the other hand, failed to shift promoters lacking the −73 to −114 region (Fig. 5, lane 25), but did shift the full-length and −114 deletion *toxT* fragments even at low membrane concentrations (Fig. 5, lanes 6 and 15). Membranes from the wild-type strain O395 also failed to interact above background with the −73 deletion fragment, even at the higher concentration, despite the fact that TcpP protein was presumably present. One possible explanation is that TcpP protein in the cell may be complexed with ToxR and, as this promoter lacks the ToxR binding site, TcpP could be sequestered away from the DNA. In the end, the biochemical evidence confirms the genetic activation data in establishing the TcpP binding site as 3′ to the

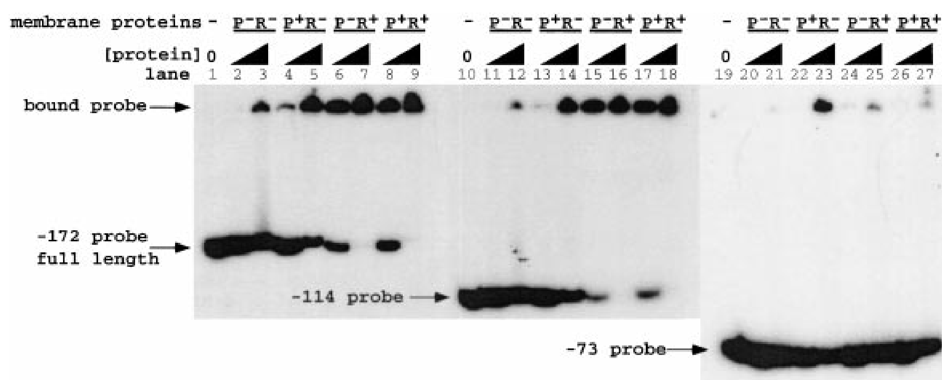


Fig. 5. *toxT* promoter deletion gel mobility shift assays. Increasing amounts of cell membrane derived from *V. cholerae* strains was mixed with end-labelled *toxT* probes for 30 min at 30°C and subjected to non-denaturing PAGE. Protein–probe complexes were retained in the well, whereas promoter deletion constructs ran into the gel according to size. Protein concentrations used were 0, 2.5 or 10 mg ml^{-1} membrane protein. The presence or absence of either ToxR (R) or TcpP (P) in each membrane preparation is denoted with either '+' or '–' respectively.

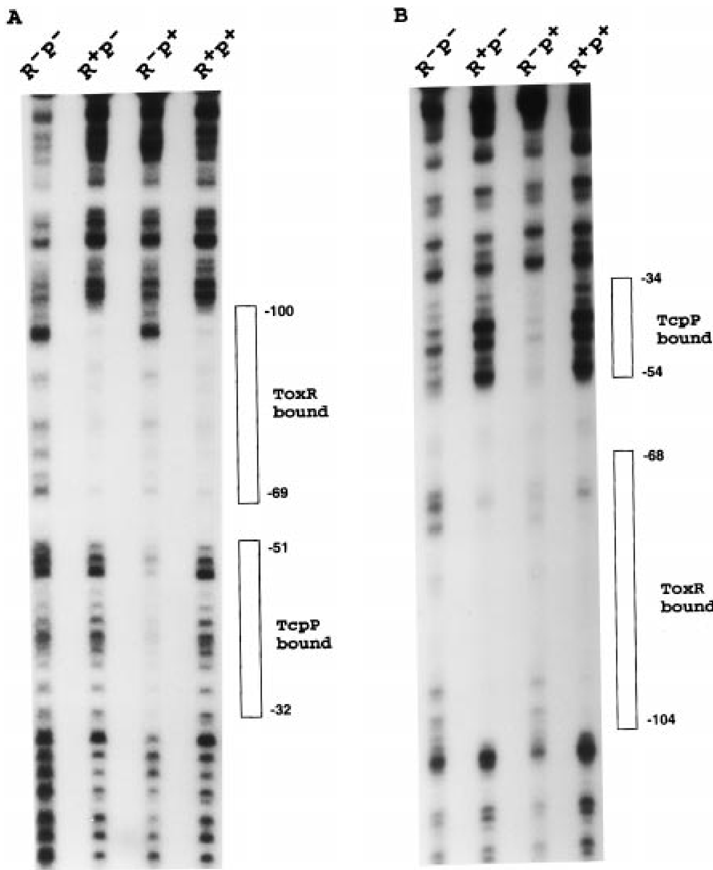


Fig. 6. DNase I footprint analysis of the *toxT* promoter. *V. cholerae* membrane extract (20 mg ml^{-1}) derived from strains expressing ToxR and/or TcpP or neither protein were mixed with the -172 to $+45$ *toxT* promoter construct in which the top strand (A) or bottom strand (B) had been end labelled. After digestion with DNase I, samples were subjected to electrophoresis in a 6% polyacrylamide denaturing gel (*Experimental procedures*).

ToxR binding site and places TcpP in close proximity to the putative RNAP consensus binding site.

TcpP-containing membranes protect a region immediately upstream of the -35 consensus site of the toxT *promoter from DNase I digestion*

In order to characterize the precise TcpP binding site within the *toxT* promoter further, membranes prepared for the EMSAs discussed above were used to generate *toxT* promoter footprints. Membranes containing TcpP, but lacking ToxR, protected a region of the top strand of the *toxT* promoter extending from nucleotides -51 to -32, including the 3′ half of the previously identified inverted repeat 1 within the *toxT* promoter (Fig. 6A, lane 3; Fig. 3A; Higgins *et al.*, 1992). Somewhat weaker protection by TcpP-containing membranes extended even further upstream to about nucleotide -80. Footprinting

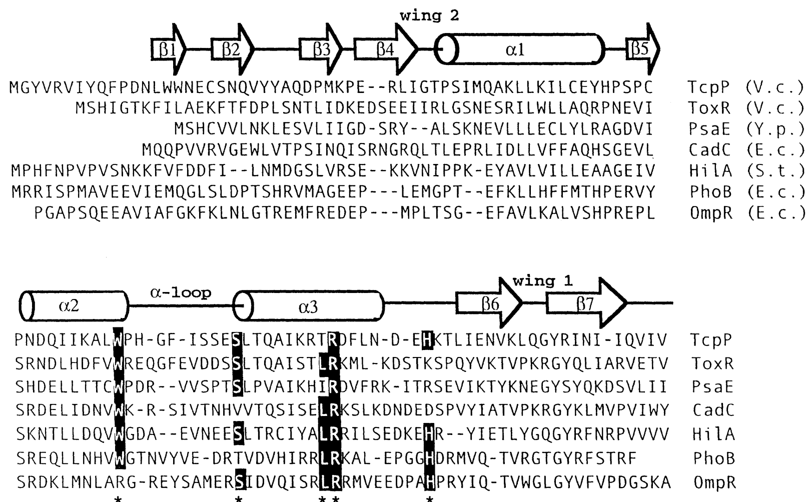


Fig. 7. Linear protein alignments of TcpP, ToxR and related proteins. Only the N-termini (TcpP, ToxR, PsaE and CadC) or C-termini (HilA, PhoB and OmpR) are shown. The secondary structure domains of OmpR, based on the crystal structure of OmpR (Martínez-Hackert and Stock, 1997a), are indicated above the sequence. TcpP residues targeted for mutagenesis are indicated by *. Proteins were aligned according to PILEUP analysis.

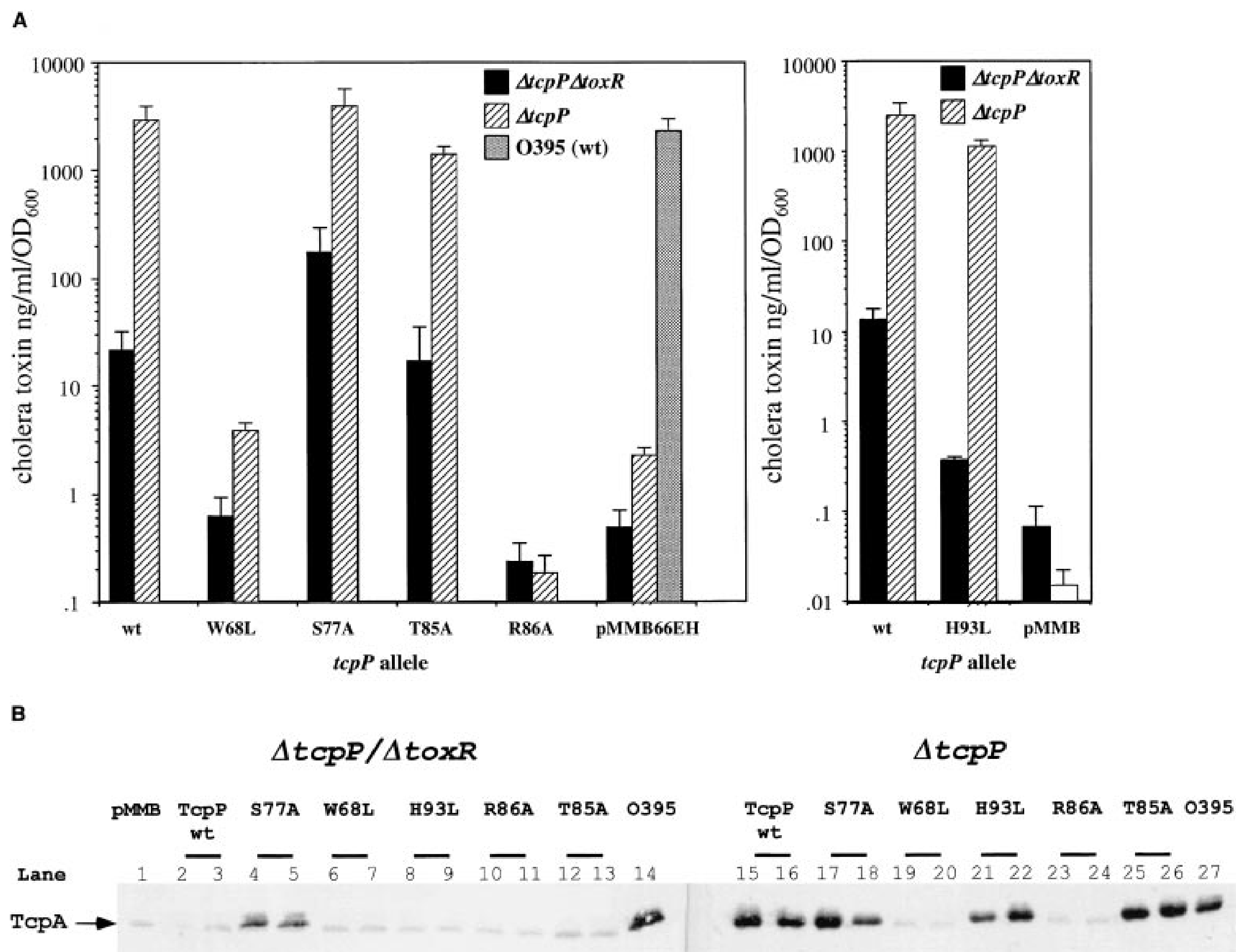
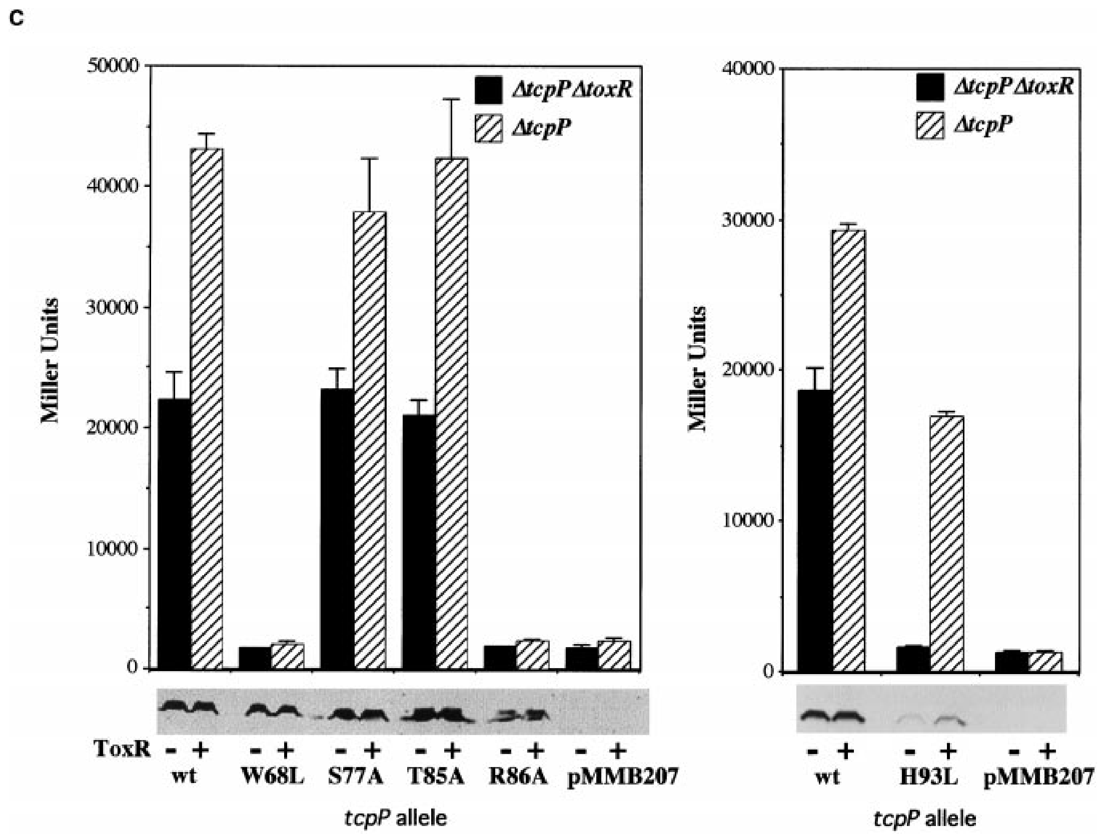


Fig. 8. Expression of cholera toxin (A), TcpA (B) or *toxT* (C) after complementation of a *ΔtcpP* strain with various *tcpP* alleles in the presence or absence of ToxR, and DNA-binding activities of TcpP mutant proteins (D).
A. *V. cholerae* cells expressing *tcpP* from an inducible plasmid (pMMB66EH) were grown overnight at 30°C in LB containing 1 mM IPTG, and supernatants were analysed for toxin production in a GM_1-ELISA assay, as described in *Experimental procedures*.
B. Cells from overnight IPTG-induced cultures were resuspended in 1 × Laemmli buffer, run on a 15% SDS–polyacrylamide gel, and TcpA was detected with an anti-TcpA antibody by Western blot analysis (*Experimental procedures*).
C. Plasmid-encoded (pMMB207), inducible *tcpP* alleles harbouring a C-terminal epitope tag were induced for 4 h in *V. cholerae ΔtcpP* or *ΔtcpPΔtoxR* in the presence of a plasmid carrying a *toxT–lacZ* fusion reporter (−172 to +45 promoter). Western blot analysis of whole-cell extracts are reported below β-galactosidase activities using an anti-HSV antibody to detect the levels of each epitope-tagged derivative in the absence or presence of ToxR.
D. Membranes derived from EK459 (*ΔtcpPΔtoxR*) expressing wild-type HSV-tagged TcpP or various mutants were bound to *toxT* promoter fragments using either 4 mg ml^{-1} or 10 mg ml^{-1} total membrane proteins as described in *Experimental procedures*. The −73 to +45 construct contains the TcpP binding site, whereas the −46 to +45 does not. Western blot analysis of the relative levels of each TcpP mutant used for gel shift analysis is shown under the appropriate lane. In the cases of TcpP-wt, TcpP-W68L, TcpP-S77A and TcpP-T85A, 2-mg ml^{-1} and 5 mg ml^{-1} TcpP containing membranes were doped with 2 mg ml^{-1} and 5 mg ml^{-1} negative control membranes (pMMB207) to achieve identical membrane concentrations and comparable TcpP levels. For pMMB207, TcpP-R86A and TcpP-H93L lanes, 4 mg ml^{-1} and 10 mg ml^{-1} undoped membranes were used (*Experimental procedures*).

with membranes containing either ToxR alone or ToxR and TcpP gave a *toxT* protection pattern indistinguishable from that observed previously with *E. coli* membranes containing ToxRS, protecting a region from −100 to −69 and characterized most strongly by protection of a hypersensitive site around position −92 (Fig. 6A, lanes 2 and 4; Li *et al*., 2000). Similar regions within the bottom strand of the *toxT* promoter were also protected, although the boundaries were slightly different (Fig. 6B). On the bottom strand, ToxR binding leads to protection of two hypersensitive sites centring around position −75. ToxR also appears to lead to enhanced cleavage within the TcpP binding site, suggesting that ToxR binding may lead to an alteration in promoter architecture (Fig. 6B). These data confirm the gel shift and promoter fusion studies, in which TcpP interacts with a region of the *toxT* promoter

C



D

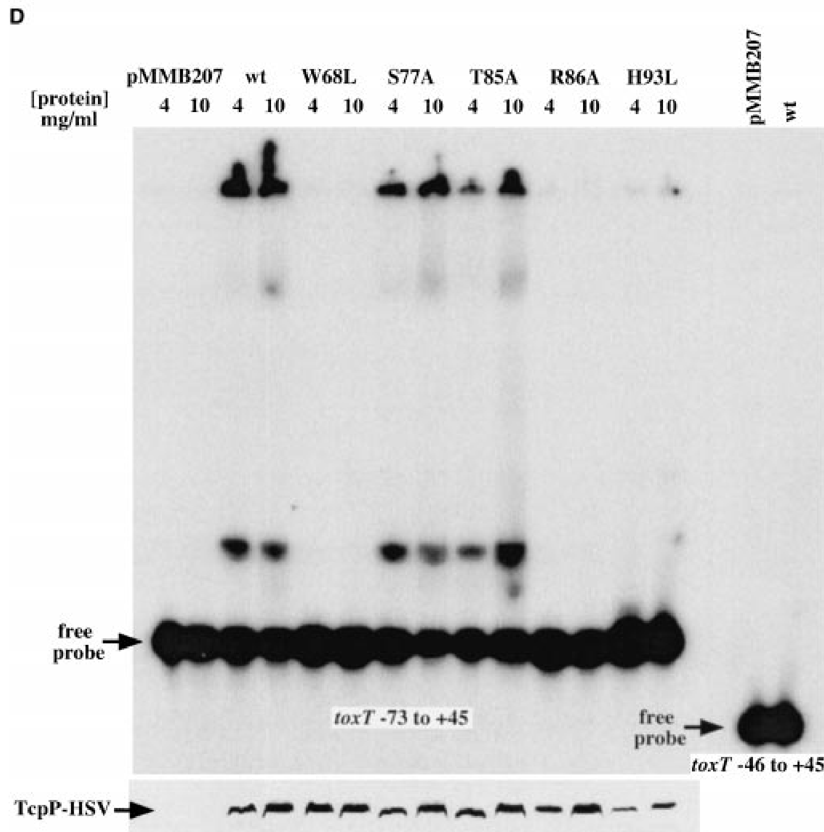


Fig. 8. Continued.

just 5′ to the RNAP consensus binding site, with ToxR binding to a region just upstream. The inability of TcpP to protect the probe in the presence of ToxR was unexpected (Fig. 6A, lane 4) and is addressed below.

ToxR expression suppresses the activation defect of a TcpP DNA-binding mutant

One possibility to account for the fact that *V. cholerae* membranes containing both TcpP and ToxR did not protect the region from −51 to −32 characteristic of TcpP alone (Fig. 6A, lane 4) is that, in the presence of ToxR, TcpP may bind DNA poorly, even though it is capable of activating transcription. This reduction in binding may result from alteration of the TcpP binding site architecture after ToxR binding upstream or ToxR–TcpP protein interactions that result in a TcpP conformation less able to bind DNA. To test this hypothesis, we generated a number of *tcpP* site-directed mutants based on mutant phenotypes of homologous OmpR-like DNA-binding proteins (Fig. 7) with the goal of obtaining derivatives defective for DNA binding or RNAP interaction.

Five TcpP derivatives were constructed by mutating a region of the protein predicted to be important for DNA binding or RNAP interaction (Ottemann *et al*., 1992; Russo *et al*., 1993; Pratt and Silhavy, 1994; Makino *et al*., 1996; Martínez-Hackert and Stock, 1997a; Fig. 7). These derivatives were expressed in *V. cholerae* in either the presence or the absence of endogenous levels of ToxR to assess their dependence on ToxR for transcriptional activation. If the above hypothesis is correct, a DNA-binding mutant of TcpP might retain its ability to activate transcription provided that ToxR is present. As an indirect measure of *toxT* expression, toxin and TcpA production resulting from *toxT* activation was assessed.

Two mutants, TcpP-W68L and TcpP-R86A, were defective for toxin and TcpA production regardless of the ToxR status of the cell (Fig. 8A and B). Although by analogy with OmpR, TcpP-W68 (*Trp-68*) resides at the edge of the α-loop of TcpP, hypothesized to be involved in direct RNAP interaction (Martínez-Hackert and Stock, 1997b), this protein was unable to bind the *toxT* promoter (Fig. 8D). Arginine-86 of TcpP was predicted to be involved in DNA binding, as some mutations in the homologous residue of ToxR disrupt promoter binding, although these defects depended on the nature of the amino acid substitution (Ottemann *et al*., 1992). Consistent with its defect in toxin and TcpA production, TcpP-R86A failed to bind *toxT* promoter DNA (Fig. 8D).

Two other mutants, TcpP-S77A and TcpP-T85A, when expressed from a plasmid, complemented both the *ΔtcpPΔtoxR* and *ΔtcpP* strains to the same extent as wild-type TcpP (Fig. 8A and B), showing that they are not defective for either ToxR-independent or ToxR-dependent activation of *toxT* transcription. In addition, both proteins maintained strong DNA-binding activity, as measured using epitope-tagged versions of each TcpP derivative (Fig. 8D, *Experimental procedures*). Consistent with the results presented above (Fig. 2), when TcpP was overexpressed, the addition of ToxR to the system enhanced both toxin expression (50- to 100-fold; Fig. 8A) and TcpA expression (Fig. 8B). TcpP-S77A appears to direct higher levels of toxin and TcpA production than wild-type TcpP in the absence of ToxR, suggesting that it may be better able to interact with the *toxT* promoter than wild-type TcpP (Fig. 8A, ToxR-independent toxin production; Fig. 8B, lanes 4 and 5).

The final derivative, TcpP-H93L, stood apart in failing to direct toxin or TcpA production in the absence of ToxR, whereas in the presence of ToxR, virulence gene expression was restored to nearly wild-type levels (Fig. 8A and B). Despite the ability of TcpP-H93L to activate *toxT* in the presence of ToxR, TcpP-H93L was almost completely defective in DNA binding (Fig. 8D). The phenotype of TcpP-H93L shows that *toxT* activation may occur if DNA binding is severely attenuated, provided that ToxR is present. This suggests that an important role for ToxR in TcpP function is to provide promoter recognition for the activation domain of TcpP to stimulate *toxT* transcription.

To measure transcriptional activation by these various TcpP derivatives directly, plasmid-expressed epitope-tagged versions of each *tcpP* allele were introduced into *V. cholerae ΔtcpP* or *ΔtcpPΔtoxR* backgrounds in the presence of a second plasmid harbouring a *toxT–lacZ* reporter construct (*toxT* promoter −172 to +45). β-Galactosidase activities directed by the various TcpP derivatives were consistent with the results seen for toxin and TcpA production, except that *toxT–lacZ* expression directed by TcpP-H93L in the presence of ToxR did not reach wild-type levels (Fig. 8C). Western analysis using antibody directed against the C-terminal epitope tag of each derivative showed TcpP-W68L, TcpP-S77A and TcpP-T85A to be as stable as wild-type TcpP (Fig. 8C). TcpP-R86A and TcpP-H93L were both less stable than wild-type TcpP, although it is clear that the level of TcpP-H93L expressed is sufficient to activate *toxT* expression strongly in the presence of ToxR (Fig. 8C). The presence of ToxR also appears to stabilize TcpP-H93L protein partially (Fig. 8C), an observation that will be discussed later.

A ToxR substitution, ToxR-G80S, results in reduced OmpU production, but wild-type toxin and TcpA levels

ToxR is believed to be the direct activator of *ompU* in *V. cholerae* (Crawford *et al*., 1998), whereas the results above suggest that TcpP is the direct activator of *toxT*. To determine whether the ability of ToxR to activate

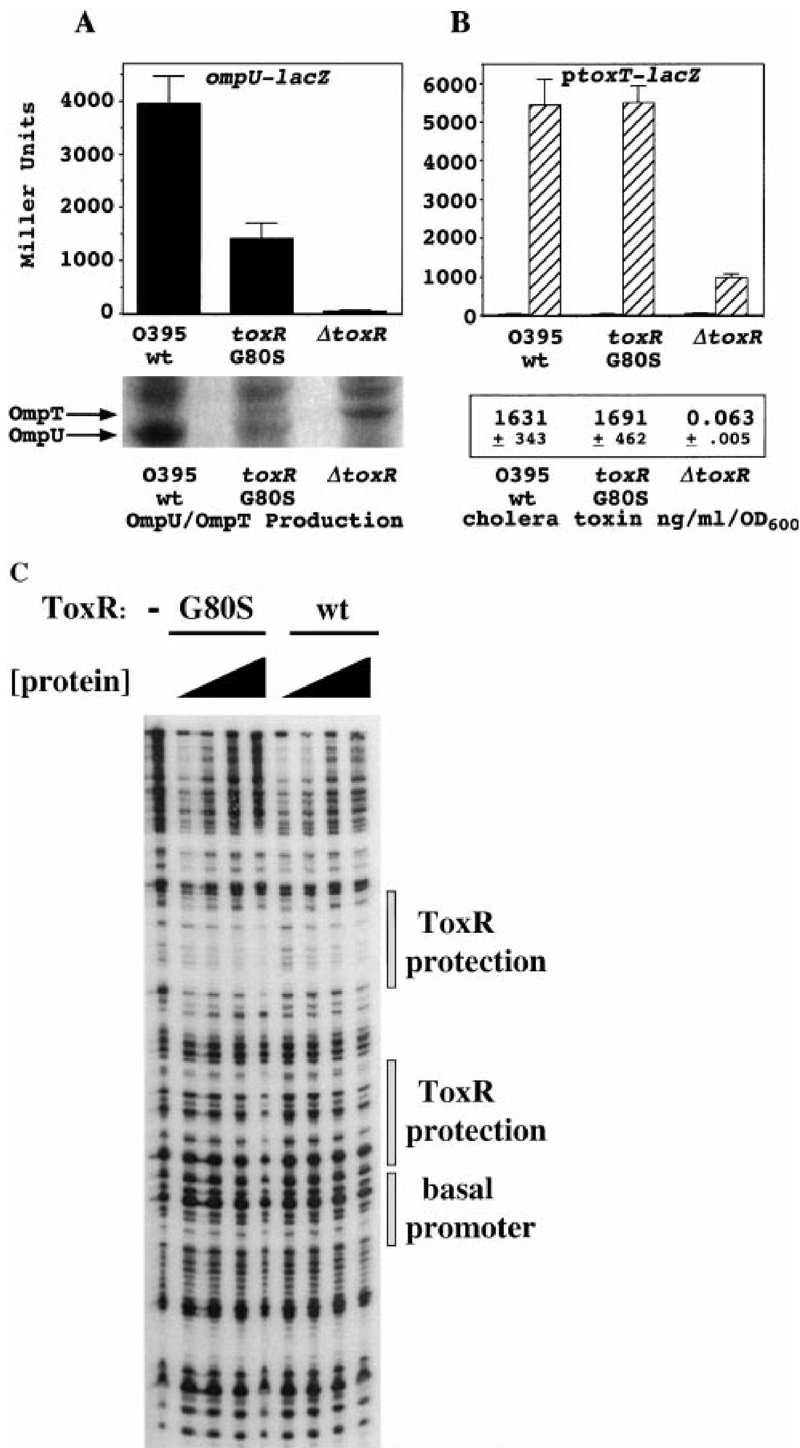


Fig. 9. Effects of a *toxR*-G80S mutation on *ompU* and *toxT* expression separates differing roles for ToxR.
A. *ompU–lacZ* expression was measured in the presence of wild-type ToxR, ToxR-G80S, or in the absence of ToxR from a chromosomal *ompU–lacZ* fusion. The corresponding levels of OmpU and OmpT from isogenic strains lacking the *ompU–lacZ* reporter are shown below the activation data.
B. *toxT–lacZ* was measured in the presence of wild-type ToxR, ToxR-G80S, or in the absence of ToxR. *toxT–lacZ* expression was from a plasmid construct (pTLI2), and the vector lacking the *toxT* promoter (pTL61T) served as the negative control. The corresponding levels of cholera toxin production, in the absence of the *toxT–lacZ* fusion plasmid, are reported below the activation data.
C. *ompU* promoter binding by wild-type ToxR and ToxR-G80S. Footprinting analysis with increasing concentrations of membrane from strains harbouring a wild-type *toxR* locus, *toxR-G80S* or Δ*toxR* was carried out on the *ompU* promoter. The concentrations of membrane used were 0, 1.25, 2.5 and 5 mg ml^{-1}. For the Δ*toxR* strain, only 5 mg ml^{-1} was used.

transcription is required at the *toxT* promoter, a *toxR* substitution mutant was constructed, *toxR*-G80S, based on the activation defect of a similar mutation characterized in the related protein, OmpR (Russo *et al*., 1993). Replacement of the wild-type *toxR* allele with the *toxR*-G80S allele (EK739) in a background harbouring an *ompU–lacZ* fusion on the chromosome led to a marked reduction in the ability of ToxR to activate the *ompU* promoter (Fig. 9A), whereas ToxR expression levels (not shown) and DNA binding were similar to wild-type levels (Fig. 9C). Decreased activation of the *ompU* promoter was also reflected in the reduced levels of OmpU in the cells and an increase in OmpT levels (Fig. 9A). *ompU–lacZ* expression was not completely abolished in the

presence of the *toxR*-G80S allele, as a *Δ toxR* derivative was decreased even further for *ompU* activation and OmpU protein levels (Fig. 9A). In contrast to the reduction in *ompU* activation, the ToxR-G80S derivative directed wild-type levels of *toxT–lacZ* expression from a plasmid harbouring the full-length *toxT* promoter fused to *lacZ* (Fig. 9B). The effect on *ompU* transcription, but not that of *toxT*, was reflected in the fact that OmpU levels were decreased, whereas toxin production by ToxR-G80S was indistinguishable from wild type (Fig. 9A and B). These results suggest that the role of ToxR at the *ompU* promoter differs from that at *toxT*. Mutations that affect the transcriptional activation domain of ToxR affect *ompU* transcription, but have no effect on *toxT* expression. Thus, rather than serving as a second activator (in direct contact with RNAP) on the *toxT* promoter, ToxR appears to use another mechanism to enhance the ability of TcpP to activate transcription.

Discussion

The current study investigated the role of both TcpP and ToxR in *toxT* promoter activation. Previous work showed that ToxR is necessary, but not sufficient, for the activation of *toxT* (Higgins and DiRita, 1994). Experiments in *E. coli* reported here confirmed the recent observation that the expression of TcpPH is sufficient to activate a *toxT–lacZ* fusion construct, and co-expression with ToxRS enhances this activation (Fig. 1A; Murley *et al*., 1999). Enhancement of TcpP-mediated *toxT* activation by ToxR has also been shown in *V. cholerae* (Fig. 3D; Häse and Mekalanos, 1998). This co-dependence of maximal *toxT* expression on TcpP and ToxR may indicate a direct interaction between these proteins, although attempts to demonstrate such an interaction have failed (C. Häse, personal communication; E. S. Krukonis, unpublished results). From these results, we conclude that TcpP is the factor previously postulated as being required for *toxT* promoter activation (Higgins and DiRita, 1994).

Although ToxR is required for TcpP-dependent activation of the *toxT* promoter in *V. cholerae* under wild-type conditions of *tcpP* expression, overexpression of *tcpPH* releases *toxT* induction from complete ToxR control. This was demonstrated most clearly by the fact that the expression of *tcpPH* alone in a *Δ tcpPΔ toxR V. cholerae* derivative (EK459) could activate a *toxT–lacZ* fusion construct (Fig. 3A). In addition, both *Δ toxR* and *Δ tcpPΔ toxR* mutant strains could be partially complemented for TcpA and toxin production by *tcpPH* overexpression (Fig. 2). Thus, ToxR may enhance the interaction of TcpP with the *toxT* promoter, an enhancement that becomes superfluous when TcpP is present at high enough concentrations, although it should be emphasized that, even under conditions of TcpP overexpression, the presence of ToxR still results in even higher levels of *toxT* expression (Figs 2 and 3D).

Our results also provide an explanation for previous observations regarding the position of the ToxR binding site on the *toxT* promoter, which is considerably upstream of the basal promoter elements (Higgins and DiRita, 1994; Li *et al*., 2000; Fig. 3C, this work). Using promoter deletion constructs fused to *lacZ*, we were able to assign the relative binding positions of TcpP and ToxR on the *toxT* promoter (Fig. 3B and C) by taking advantage of the fact that overexpression of TcpPH obviates the requirement of ToxR for activation. Both promoter activation experiments (Fig. 3B) and gel shift analysis using *V. cholerae* membranes (Fig. 5) demonstrated that TcpP interacts with a region of the *toxT* promoter in close proximity to the predicted RNAP consensus binding site. Footprinting analysis in the presence of TcpP or ToxR confirmed these results and allowed us to assign the interaction sites as −51 to −32 for TcpP and −100 to −69 for ToxR. This ToxR binding site is identical to that found using membranes from *E. coli* expressing ToxRS as the source of ToxR (Li *et al*., 2000). The region protected from DNase I digestion by TcpP may extend further upstream to about nucleotide −80 (Fig. 6), but such protection is weaker. This may explain why a *toxT* promoter truncated at −73 can be activated by TcpP, yet longer constructs are even more strongly activated (Fig. 3). That the TcpP and ToxR binding sites appear to overlap in the region from nucleotides −69 to −80 of the *toxT* promoter may reflect the fact that both proteins contain similar OmpR-like DNA-binding domains. As the promoter binding position of TcpP is similar to that of several other transcriptional activators (Collado-Vides *et al*., 1991) and given the similarity between TcpP and OmpR-like proteins, we propose that TcpP activates transcription by contacting RNAP directly (Ishihama, 1992; Rhodius and Busby, 1998).

The role of ToxR may be reflected in the fact that, at wild-type levels of expression, ToxR interacts with the *toxT* promoter more readily than does TcpP (Fig. 4). Based on this observation, we propose a model in which TcpP interacts weakly with its promoter binding site, and ToxR serves to recruit TcpP or stabilize the poor promoter interaction of TcpP (Fig. 10). This is consistent with the observation that, at high concentrations, TcpP (a relatively inefficient promoter-binding protein) can activate transcription in the absence of ToxR (Fig. 3B). ToxR may enhance the activity of TcpP by a direct protein–protein interaction. Although ToxR–TcpP interaction has not been shown directly, we did observe that membranes containing ToxR and TcpP failed to bind to the −73 *toxT* promoter deletion construct, whereas those containing TcpP alone bound well (Fig. 5, lanes 23 and 27). This may indicate that ToxR and TcpP interact away from the

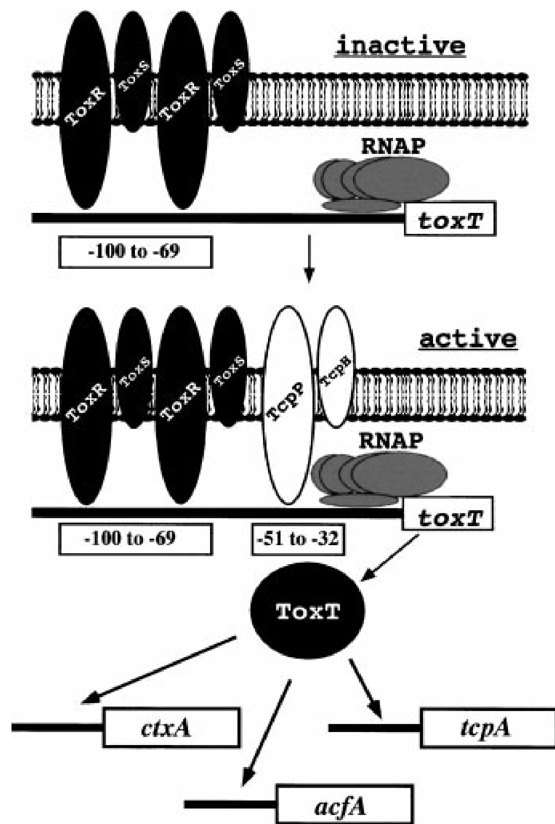


Fig. 10. Model for *toxT* promoter activation. Both ToxR and TcpP are required for the activation of the *toxT* promoter when these proteins are expressed at physiological levels. ToxR is produced constitutively in *V. cholerae* and binds the *toxT* promoter from −100 to −69. TcpP production is environmentally regulated. Once synthesized, TcpP binds to promoter sequences between −51 and −32. As ToxR interacts more readily with the *toxT* promoter, it may serve to recruit TcpP to a relatively weak binding site, thus allowing contact with RNAP and initiation of transcription. Both ToxR and TcpP require accessory proteins (ToxS and TcpH respectively) for maximal activity. ToxR also protects a larger region of the *toxT* promoter from DNase I digestion than TcpP and may thus bind to multiple sites upstream of TcpP.

promoter, and deletion of the ToxR binding site from the *toxT* promoter results in sequestration of TcpP away from its potential promoter site. In the case of the DNase I footprinting results (Fig. 6), in which the ToxR binding site is intact, but TcpP fails to protect its binding site in the presence of ToxR, the ToxR–TcpP association may alter the conformation of TcpP, such that it is competent for transcriptional activation, but less able to bind DNA. The ability of ToxR to stabilize partially a particular TcpP derivative (TcpP-H93L; Fig. 8C) also suggests that the proteins may interact.

The ratio of TcpP to ToxR also appears to be critical for *toxT* activation, as overexpression of ToxRS in wild-type *V. cholerae* (O395) leads to a marked reduction in the amount of toxin produced (404 ng ml^{-1}/OD_{600} in the wild type versus 14.8 ng ml^{-1}/OD_{600} with ToxRS overexpression). This reduction is also consistent with an interaction between ToxR and TcpP, and could result from titration of TcpP away from the *toxT* promoter by excess unbound ToxR. Alternatively, it may represent occlusion of the TcpP binding site by ToxR. Plasmid-encoded *toxR* complements the *ΔtoxR* mutant used in this study (EK307) to a higher degree at low levels of induction (10 μM IPTG) than at higher levels (1 mM), also suggesting that overexpression of ToxR is detrimental to *toxT* activation (E. S. Krukonis and A. Crawford, unpublished observations).

A number of *tcpP* and *toxR* point mutants were generated with the aim of isolating some defective for DNA binding or transcriptional activation activity. According to the hypothesis that ToxR recruits TcpP to the *toxT* promoter via a protein–protein interaction, TcpP DNA-binding mutants would be rescued for activation of *toxT* by being co-expressed with ToxR. In this scenario, ToxR would provide the promoter recognition function of the activation system, whereas TcpP would contact RNAP directly. One TcpP derivative tested, TcpP-H93L, behaves precisely as predicted from this model. When over-expressed alone in *V. cholerae*, TcpP-H93L cannot direct the expression of cholera toxin or TcpA; however, when co-expressed with endogenous levels of ToxR, this TcpP variant directs the expression of near wild-type levels of these two ToxT-dependent products (Fig. 8A and B). As TcpP-H93L is defective for DNA binding (Fig. 8D), ToxR appears to provide promoter recognition for *toxT* activation. Activation of the *toxT* promoter by TcpP-H93L in the presence of ToxR does not reach wild-type levels (Fig. 8C), although toxin and TcpA production does. This may indicate that, after a certain cellular concentration of ToxT is achieved, further *toxT* activation does not lead to increased toxin and TcpA production. Our understanding of the mechanism by which ToxR rescues *toxT* activation by TcpP-H93L is complicated by the fact that ToxR partially stabilizes the relatively unstable TcpP-H93L protein (Fig. 8C); however, ToxR is clearly able to facilitate transcription activation activity by a TcpP mutant whose ability to bind DNA has been severely diminished. This suggests that ToxR may enhance the ability of TcpP to interact with the *toxT* promoter, thus positioning TcpP in close proximity to RNAP and leading to transcriptional activation. The fact that ToxR partially stabilizes TcpP-H93L also suggests that these proteins may interact.

Two other interesting *tcpP* mutants are TcpP-W68L and TcpP-R86A, both of which fail to activate transcription even in the presence of ToxR (Fig. 8A–C). In addition, both mutants fail to bind the *toxT* promoter. Why TcpP-H93L, but not TcpP-W68L or TcpP-R86A, can be partially rescued for *toxT* activation by ToxR is unclear, but the latter two mutants may affect DNA binding as well as ToxR–TcpP interaction or the ability of TcpP to recognize

Table 1. Strains, plasmids and oligonucleotides.

Name	Description	Reference/source
Strain		
V. cholerae		
O395	Wild type, Str^R	Laboratory collection
EK307	O395 *Δ toxR*	This study
RY1	O395 *Δ tcpP*	Yu and DiRita (1999)
EK459	O395 *Δ toxR Δ tcpP*	This study
EK385	O395 *lacZ::ompU-lacZ*	This study
EK410	O395 *Δ toxR lacZ::ompU-lacZ*	
EK739	O395 *toxR*-G80S *lacZ::ompU-lacZ*	This study
EK698	O395 *toxR*-G80S	This study
E. coli		
AC174	*IN (rrnD-rrnE)1 Δ (lac)X74 rpsL galK2 recD1903::*Tn*10d-Tet trpDC700::putPA1303 ::[KanS-CamR-lac] [KanR-pompU-lacZ]*	Crawford *et al.* (1998)
BMH71-18	*thi supE Δ (lac-proAB) mutS::*Tn*10* (Tet)	Promega
DH5α	*supE44 ΔlacU169(F80lacZDM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory collection
DH92	*IN (rrnD-rrnE)1 Δ (lac)X74 rpsL galK2 recD1903::*Tn*10d-Tet trpDC700::putPA1303 ::[KanS-CamR-lac] [KanR-ptoxT-lacZ]*	Higgins and DiRita (1994)
SM10λpir	*thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu* Km^R *(λ pir)*	Laboratory collection
MM294	F^- *endA1 hsdR17* $(r_K^- m_K^-)$ *supE44 thi-1 relA1 rfbD1 spoT1* pRK2013	Laboratory collection
VJ787	*IN (rrnD-rrnE)1 Δ (lac)X74 rpsL galK2 recD1903::*Tn*10d-Tet trpDC700::putPA1303 ::[KanS-CamR-lac] [KanR-pctxA-lacZ]*	Bock and V. J. DiRita (unpublished)
Plasmids		
pACYC184	Expression vector	Laboratory collection
pVJ21	pACYC184-*toxRS*	DiRita *et al.* (1991)
pMMB66EH	Expression vector	Fürste *et al.* (1986)
pMMB207	Expression vector	Morales *et al.* (1991)
pEK18	p6891MCS-*ompU–lacZ*	This study
pEK25	pMMB66EH-*tcpPH* Amp^R	This study
pEK32	pMMB207-*tcpPH* Cm^R	This study
pEK38	pMMB66EH-*tcpP* wt	This study
pEK38-W68L	pMMB66EH-*tcpP*-W68L	This study
pEK38-S77A	pMMB66EH-*tcpP*-S77A	This study
pEK38-T85A	pMMB66EH-*tcpP*-T85A	This study
pEK38-R86A	pMMB66EH-*tcpP*-R86A	This study
pEK38-H93L	pMMB66EH-*tcpP*-H93L	This study
pAlter	Mutagenesis vector	Promega
pTL61T	Promoterless *lacZ* vector	Linn and Pierre (1990)
pTLI2	pTL61T–p*toxT* (−172 to +45)	Higgins and DiRita (1994)
pKAS32	Suicide plasmid	Skorupski and Taylor (1996)
pKAS32-*Δ toxR*	*toxR* deletion suicide plasmid	This study
pKAS32-*Δ tcpP*	*tcpP* deletion suicide plasmid	Yu and DiRita (1999)
pKAS32-*toxR*-G80S	*toxR* allelic exchange suicide plasmid	This study
pSK-*tcpP*-HSV	HSV epitope-tagged *tcpP* in pBluescript	This study
pEK41	pMMB207-tcpP-HSV	This study
pEK41-W68L	pMMB207-*tcpP*-W68L-HSV	This study
pEK41-S77A	pMMB207-*tcpP*-S77A-HSV	This study
pEK41-T85A	pMMB207-*tcpP*-T85A-HSV	This study
pEK41-R86A	pMMB207-*tcpP*-R86A-HSV	This study
pEK41-H93L	pMMB207-*tcpP*-H93L-HSV	This study
Oligonucleotides		
toxR 5′ *Bam*HI	5′-GGGGATCCATTGAGACTCAATGGAATTACCTTGATG-3′	This study
toxRS 3′ *Bam*HI	5′-GGGGATCCCAATTAAATCACGCACTCTTTGCTTCA-3′	This study
Δ toxR SOE top	5′-CTCAAAAGAGATATCGATGCAAAATAGACACATC-3′	This study
Δ toxR SOE bottom	5′-GATGTGTCTATTTTGCATCGATATCTCTTTTGAG-3′	This study
tcpP W68L	5′-CAAATAATTAAAGCACTTTTGCCTCATGGATTTATC-3′	This study
tcpP S77A	5′-GGATTTATCAGCTCTGAAGCTCTAACTCAGGCAATC-3′	This study
tcpP T85A	5′-CAGGCAATCAAAAGAGCTCGTGATTTTTTGAATG-3′	This study
tcpP R86A	5′-GGCAATCAAAAGAACCGCGGATTTTTTGAATGATG-3′	This study
tcpP H93L	5′-GATTTTTTGAATGATGAACTTAAGACGTTGATCG-3′	This study
toxR G80S	5′-GTTTGGCGAGAGCAAAGTTTTGAAGTCGATG-3′	This study
tcpP 5′	5′-GGGGTACCGATAACTTTGCAACCGTT-3′	Yu and DiRita (1999)
tcpP 3′	5′-TCCCCGCGGGACGATCTCAATACAACT-3′	Yu and DiRita (1999)
ompU–lacZ 5′	5′-CCCCGAATTCCCTAAATCGGGTCG-3′	Crawford *et al.* (1998)
ompU–lacZ 3′	5′-CCCCGAATTCACAGGAGCACGATC-3′	This study
tcpP 5′*Eco*RI	5′-GGGAATTCGTAAAGTAATGGGGTATG-3′	This study
tcpP 3′ *Xho*I	5′-CCTGTAGCGCTCGAGTTAATTTTTTGTGCATTC-3′	This study
tcpH 3′ *Bam*HI	5′-GGGGATCCTACTCGTCACAGACT-3′	This study

Table 1. *continued*

Name	Description	Reference/source
*tcpP Sma*I-HSV	5′-GGGGCATTTTTTGTGCATTCTAATGTC-3′	This study
toxR 5′ G80S	5′-GACTTTGTTTGGCGAGAGCAAA-3′	This study
toxR 5′ G80wt	5′-GACTTTGTTTGGCGAGAGCAAG-3′	This study
lacZ 5′	5′-GATCTGAAGTCATCCGTAATC-3′	This study
lacZ 3′	5′-TTAAGGCTCTCTGGCTTATTG-3′	This study

a ToxR-induced *toxT* promoter architectural change. It should be noted that TcpP-R86A is somewhat unstable based on the detection of an HSV epitope-tagged version by Western blot (Fig. 8C). However, TcpP-H93L is even more unstable than TcpP-R86A (Fig. 8C), and its reduced protein levels are sufficient to activate *toxT* in the presence of ToxR. Thus, if TcpP-R86A had significant ToxR-dependent *toxT* activation activity, it should have been observed.

Finally, a derivative of ToxR, ToxR-G80S, was defective for *ompU* activation, but maintained wild-type levels of *toxT–lacZ* activation and toxin production (Fig. 9). This allows us to differentiate between the role that ToxR plays on promoters such as *ompU*, in which it is the *de facto* activator, from its role on the *toxT* promoter, in which it serves to enhance the ability of TcpP to activate transcription. The fact that the transcriptional activation activity of ToxR is dispensable for *toxT* activation is consistent with the role of ToxR as an enhancer of TcpP function and not as an activator in the usual sense of the term.

Taken together, the data presented here clearly indicate a role for both TcpP and ToxR in activation of the *toxT* promoter. In our current model, TcpP binds the *toxT* promoter at a polymerase-proximal site encompassing nucleotides −51 to −32. ToxR occupies a more distal binding site from −100 to −69, possibly involving more than one ToxR molecule (DiRita and Mekalanos, 1991; Harlocker *et al.*, 1995). We propose that the role of ToxR in activation is to recruit the weaker binding TcpP to the TcpP promoter binding site, thereby situating TcpP in close enough proximity to RNAP to initiate transcription (Fig. 10). Whether this recruitment occurs by direct protein–protein interaction or alteration of the *toxT* promoter DNA to allow TcpP access to a position that is competent for transcriptional activation remains to be determined, as does the nature of the putative interaction of TcpP with RNAP. As ToxR appears to be produced under various environmental conditions in *V. cholerae* (DiRita *et al.*, 1996), whereas TcpP is transcriptionally regulated in response to both temperature and pH (Thomas *et al.*, 1995; Carroll *et al.*, 1997; Häse and Mekalanos, 1998; Skorupski and Taylor, 1999), we hypothesize that ToxR constitutively binds the *toxT* promoter, but activation does not ensue until TcpP is synthesized. At that point, ToxR directs TcpP to its position on the *toxT* promoter to contact RNAP and activate transcription (Fig. 10). Thus, the *V. cholerae* virulence cascade is set in motion.

Experimental procedures

Bacterial strains and plasmids

All *E. coli* and *V. cholerae* strains, plasmids and oligonucleotides used in this study are listed in Table 1. The construction of *E. coli* strain DH92 harbouring a *toxT–lacZ* fusion on the chromosome has been described previously (Higgins and DiRita, 1994). Strains were grown in LB medium at 30°C or 37°C. Strains were maintained in LB medium containing 20% glycerol at −70°C. Antibiotics were used at the following concentrations: streptomycin 100 μg ml^{-1}; ampicillin 100 μg ml^{-1}; tetracycline 12.5 μg ml^{-1}; chloramphenicol 25 μg ml^{-1}; and kanamycin 40 μg ml^{-1}. DNA was transferred into *V. cholerae* by electroporation (2.2 kV, 200 W) using an *E. coli* Pulsor (Bio-Rad) or by conjugation using an *E. coli* strain harbouring the mobilization plasmid pRK2013. *E. coli* strains were transformed by standard methods (Sambrook *et al.*, 1989).

Construction of ΔtoxR, ΔtcpP and ΔtoxRΔtcpP *strains*

The *ΔtoxR* strain EK307 was constructed using the suicide vector pKAS32 (Skorupski and Taylor, 1996), into which a DNA fragment obtained by SOEing polymerase chain reaction (PCR) (Higuchi, 1990) containing 233 nucleotides upstream of the *toxR* start codon fused to the start codon of *toxS* and 805 downstream nucleotides had been inserted. This *toxR* deletion fragment was generated by first amplifying the N-terminal end of *toxR* up to the start codon and engineering a 5′ *Bam*HI site using primers *toxR* 5′*Bam*HI and *ΔtoxR* SOE bottom (Table 1). Next, the *toxS* and downstream sequences were amplified, and a 3′ *Bam*HI site was engineered using oligos *toxRS* 3′*Bam*HI and *ΔtoxR* SOE top. Oligos *ΔtoxR* SOE bottom and *ΔtoxR* SOE top overlap with one another and encode the fusion junction. After the separate PCR reactions, the products were cleaned with Qiex beads (Qiagen), and a final PCR reaction was performed using the primers *toxR* 5′*Bam*HI and *toxRS* 3′*Bam*HI to generate a fragment with the internal *toxR* deletion. This product was digested with *Bam*HI, inserted into *Bgl*II-cut pKAS32 and mated into *V. cholerae* strain O395 using the *E. coli* strain SM10λ*pir* by filter conjugation. The *ΔtcpP* strain RY1 was constructed similarly as described by Yu and DiRita (1999). The *ΔtoxRΔtcpP* strain EK459 was constructed by mating strain EK307 (*ΔtoxR*) with SM10λ*pir*

harbouring the pKAS32-Δ*tcpP* plasmid. Recombination and loss of the wild-type allele was confirmed by PCR analysis using primers *toxR* 5′*Bam*HI/*toxRS* 3′*Bam*HI *(toxR)* or *tcpP* 5′/*tcpP* 3′ *(tcpP)*. PCR reactions for cloning were performed with the Expand High Fidelity PCR system (Boehringer Mannheim).

DNA gel mobility shift assays

Membrane extracts derived from the various *V. cholerae* strains were prepared as described previously (Miller *et al*., 1987) and stored at −70°C in 25% sucrose with 5 mM EDTA. Protein concentrations were determined using the Bio-Rad protein quantification assay. *toxT* promoter fragments were end labelled by digesting ≈10–20 μg of the plasmid pTLI2 (or appropriate deletion derivatives) with *Sal*I in a 30 μl volume, followed by a fill-in reaction using 5 units of Klenow DNA polymerase (New England Biolabs), 2 mM dGTP, dATP and dTTP as well as 30 μCi of [α-^{32}P]-dCTP (Amersham). After a 15 min, 70°C heat inactivation of the Klenow enzyme, the promoter fragment was liberated by a final digestion with *Bam*HI. The promoter fragment was then purified by PAGE. Probe was eluted from the gel overnight at 37°C and adjusted to a concentration of 20 000 c.p.m. μl^{-1}. Various concentrations of membrane were then mixed with 3000 c.p.m. or 20 000 c.p.m. of probe in the presence of 10 μg ml^{-1} sheared salmon sperm DNA and 10 mM Tris, pH 7.4, 1 mM EDTA, 5 mM NaCl, 50 mM KCl, 50 μg ml^{-1} BSA in a 20 μl final volume. Binding reactions were performed at 30°C for 30 min, and samples were run on a 6% polyacrylamide non-denaturing gel that had been prerun with 20 μl of 5% thioglycolic acid in each well to prevent membrane–DNA complexes from entering the gel. DNA binding was determined by comparing the amount of free probe and the amount of probe retained in the well. In the case of epitope-tagged TcpP derivatives, Western blots were used to determine the amount of TcpP in each membrane preparation from the EK459 *V. cholerae* background. Membrane concentrations were adjusted to give equivalent levels of TcpP in the binding reactions. For TcpP-wt, TcpP-W68L, TcpP-S77A and TcpP-T85A, 2 mg ml^{-1} and 5 mg ml^{-1} membranes were used. For EK459 + pMMB207 (empty vector), TcpP-R86A and TcpP-H93L, 4 mg ml^{-1} and 10 mg ml^{-1} were used. In order to have equivalent amounts of total membrane proteins in the binding reactions, an additional 2 mg ml^{-1} and 5 mg ml^{-1} EK459 + pMMB207 membranes were added to the first four reactions to achieve a final concentration of 4 mg ml^{-1} and 10 mg ml^{-1} total protein. Western blots were then used to show the relative levels of TcpP-HSV present in each binding reaction.

DNase I footprinting analysis

DNase I footprinting reactions were performed as described previously (Crawford *et al*., 1998) with the exception that 20 mg ml^{-1} *V. cholerae* membranes were used rather than *E. coli*. Reactions were digested for 2 min at room temperature using 0.1 units of DNase I. *toxT* promoter fragments were cut from the vector pTLI2 with either *Sal*I (bottom strand labelling) or *Bam*HI (top strand labelling) first, filled in and then cut with *Bam*HI or *Sal*I, respectively, to generate differentially labelled strands. Labelled probe (70 000 c.p.m.) was used in each 70 μl reaction.

Construction of tcpP *expression plasmids*

The *tcpP* gene or *tcpPH* operon was amplified from chromosomal DNA derived from O395 (Classical) or E7946 (El Tor) *V. cholerae* strains using the Expand High Fidelity PCR system (Boehringer Mannheim) and the oligonucleotides *tcpP* 5′*Eco*RI/*tcpP* 3′*Xho*I or *tcpP* 5′*Eco*RI/*tcpH* 3′*Bam*HI. After PCR amplification, products were digested with *Eco*RI–*Xho*I or *Eco*RI–*Bam*HI and ligated into *Eco*RI–*Sal*I or *Eco*RI–*Bam*HI cut pMMB66EH. TcpP(H) inserts were subsequently moved to pMMB207 as *Eco*RI–*Pst*I or *Eco*RI–*Bam*HI fragments to allow inducible expression in a chloramphenicol-resistant vector. pMMB66EH and pMMB207 were kindly supplied by Michael Bagdasarian (Michigan State University).

Construction of tcpP *and* toxR *site-directed mutants*

Site-directed mutants in *tcpP* were generated by cloning the *tcpP* gene into the mutagenesis vector pAlter (Promega), annealing oligonucleotides containing the changes of interest to the wild-type sequence and carrying out a polymerization and ligation reaction according to the manufacturer's instructions. Mutants were confirmed by DNA sequencing (Sequenase; US Biochemicals). Mutant alleles were then liberated from pAlter as an *Eco*RI–*Pst*I fragment and cloned into an *Eco*RI–*Pst*I-digested expression vector pMMB66EH (Fürste *et al*., 1986). Mutant alleles were mated into *V. cholerae* derivatives and used to measure complementation of a chromosomal Δ*tcpP* allele. The *toxR*-G80S allele was constructed similarly in pAlter. After sequencing, *toxR*-G80S was liberated as a *Bam*HI fragment and ligated into *Bgl*II-digested suicide plasmid pKAS32. The resulting pKAS-*toxR*-G80S was transformed into *E. coli* SM10λpir and then mated into O395 or EK385 (O395 *lacZ::ompU–lacZ*) by filter conjugation. Replacement of the wild-type *toxR* locus was confirmed by selective PCR using 5′ oligonucleotides with a 3′ end corresponding to the site of the G80S mutation (5′*toxR*-G80S or 5′*toxR*-G80wt) and the 3′ oligo *toxRS* 3′*Bam*HI with annealing at 55°C. A carboxy-terminal HSV epitope tag was added to various *tcpP* alleles by amplifying the wild-type *tcpP* allele by PCR using primers *tcpP* 5′*Eco*RI and *tcpP* 3′*Sma*I, which remove the TAA stop codon and provide a portion of a *Sma*I site. This PCR product was treated with mung bean nuclease (New England Biolabs) to ensure blunt ends, cut with *Eco*RI and ligated into *Eco*RI–*Sma*I-digested pMMB66EH. The resulting plasmid was digested with *Sma*I, and an HSV epitope-tagging oligo was inserted by ligation (Novagen). The wild-type HSV-tagged *tcpP* allele was moved into pBluescript SK (Stratagene) as an *Eco*RI–*Pst*I fragment, and then various *tcpP* mutant alleles were ligated into this backbone as *Eco*RI–*Bst*EII cassettes, replacing the 5′ end of the wild-type *tcpP* gene. The various *tcpP* mutants were then shuttled as *Eco*RI–*Pst*I fragments into the inducible expression plasmid pMMB207. Expression was assessed after growth in 1 mM IPTG for various times by Western blotting using an

anti-HSV monoclonal antibody (Novagen) followed by an anti-mouse 2° antibody conjugated to alkaline phosphatase (AP; Gibco BRL).

Measurement of toxT–lacZ*,* ompU–lacZ *or* ctxA–lacZ *activation*

Cells were grown overnight at 37°C (*E. coli*) or 30°C (*V. cholerae*) in LB, diluted 1:100 in fresh LB with 1 mM IPTG, where appropriate, and grown for an additional 3 h at 37°C (*E. coli*) or 4 h at 30°C (*V. cholerae*). Samples of 20–100 μl of culture were used for measurement of β-galactosidase activity as described previously (Miller, 1972), and the OD_{600} was determined and used to normalize cultures for subsequent Western blot analysis of ToxR or TcpA expression and calculation of cholera toxin expression.

Measurement of cholera toxin production

Cholera toxin levels of various derivatives were determined by growing cultures overnight at 30°C in LB with 1 mM IPTG where appropriate. The culture (1 ml) was microcentrifuged for 5 min, and the resulting supernatant was diluted in a $GM\text{-}_1$ ganglioside-coated microtitre plate as described previously (Svennerholm and Holmgren, 1978). Toxin binding was revealed with polyclonal anti-CtxB antibodies provided by Dr Michael Bagdasarian (Michigan State University) at a 1:1000 dilution, followed by an anti-rabbit AP-conjugated 2° antibody at 1:2000 dilution (Gibco BRL). The chromagenic substrate ONPG (Sigma) was used at 4 mg ml^{-1}, and Abs405 was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader.

Measurement of TcpA and ToxR production

V. cholerae cells were grown overnight in LB at 30°C with 1 mM IPTG if appropriate. Cells (1 ml) were microcentrifuged and resuspended in 200–500 ml of sample buffer (Sambrook *et al*., 1989). Cells were resuspended in appropriate volumes to normalize for different OD_{600} readings. Cells were boiled for 5 min, and equal volumes of cell extract were loaded for SDS–PAGE analysis. TcpA was detected by Western blot analysis using anti-TcpA polyclonal antibodies generously provided by Dr Ron Taylor (Dartmouth Medical School) at 1:100 000 dilution, and ToxR was detected using polyclonal anti-ToxR antibodies kindly supplied by Dr John Mekalanos (Harvard Medical School) at 1:1000 dilution.

Detection of OmpU or OmpT protein

V. cholerae cultures were grown for various times, then 1 ml of culture was pelleted and resuspended in 1 × sample buffer (Sambrook *et al*., 1989). The volume of resuspension was adjusted to normalize for culture OD_{600}. Whole-cell pellets were boiled for 5 min and run on a 12% polyacrylamide gel at 150 V, followed by staining with Coomassie brilliant blue. OmpU (40 kDa) and OmpT (38 kDa) are predominant bands in the whole-cell lysates.

Acknowledgements

We would like to thank Adam Crawford, Stephen Cendrowski, Claudia Häse and Ana Coehlo for many helpful discussions of this work. This work was supported by grants AI31645 and AI45125 to V.J.D. E.S.K was supported by NRSA grant AI09942. DNA sequence analysis was supported in part by grant M01-RR 00042 to the University of Michigan Clinical Research Center.

References

Carroll, P.A., Tashima, K.T., Rogers, M.B., DiRita, V.J., and Calderwood, S.B. (1997) Phase variation in *tcpH* modulates expression of the ToxR regulon in *Vibrio cholerae*. *Mol Microbiol* **25**: 1099–1111.

Chakrabarti, S.R., Chaudhuri, K., Sen, K., and Das, J. (1996) Porins of *Vibrio cholerae*: purification and characterization of OmpU. *J Bacteriol* **178**: 524–530.

Champion, G.A., Neely, M.N., Brennan, M.A., and DiRita, V.J. (1997) A branch in the ToxR regulatory cascade of *Vibrio cholerae* revealed by characterization of *toxT* mutant strains. *Mol Microbiol* **23**: 323–331.

Collado-Vides, J., Magasanik, B., and Gralla, J.D. (1991) Control site location and transcriptional regulation in *Escherichia coli*. *Microbiol Rev* **55**: 371–394.

Crawford, J.A., Kaper, J.B., and DiRita, V.J. (1998) Analysis of ToxR-dependent transcription activation of *ompU*, the gene encoding a major envelope protein in *Vibrio cholerae*. *Mol Microbiol* **29**: 235–246.

DiRita, V.J., and Mekalanos, J.J. (1991) Periplasmic interaction between two membrane regulatory proteins, ToxR and ToxS, results in signal transduction and transcriptional activation. *Cell* **64**: 29–37.

DiRita, V.J., Parsot, C., Jander, G., and Mekalanos, J.J. (1991) Regulatory cascade controls virulence in *Vibrio cholerae*. *Proc Natl Acad Sci USA* **88**: 5403–5407.

DiRita, V.J., Neely, M., Taylor, R.K., and Bruss, P.M. (1996) Differential expression of the ToxR regulon in classical and El Tor biotypes of *Vibrio cholerae* is due to biotype-specific control over *toxT* expression. *Proc Natl Acad Sci USA* **93**: 7991–7995.

Elliott, T. (1992) A method for constructing single-copy *lac* fusions in *Salmonella typhimurium* and its application to the *hemA–prfA* operon. *J Bacteriol* **174**: 245–253.

Fürste, J.P., Pansegrau, W., Frank, R., Blöcker, H., Scholz, P., Bagdasarian, M., *et al*. (1986) Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. *Gene* **48**: 119–131.

Harlocker, S.L., Bergstrom, L., and Inouye, M. (1995) Tandem binding of six OmpR proteins to the *ompF* upstream regulatory sequence of *Escherichia coli*. *J Biol Chem* **270**: 26849–26856.

Häse, C.C., and Mekalanos, J.J. (1998) TcpP protein is a positive regulator of virulence gene expression in *Vibrio cholerae*. *Proc Natl Acad Sci USA* **95**: 730–734.

Higgins, D.E., and DiRita, V.J. (1994) Transcriptional control of *toxT*, a regulatory gene in the ToxR regulon of *Vibrio cholerae*. *Mol Microbiol* **14**: 17–29.

Higgins, D.E., Nazareno, E., and DiRita, V.J. (1992) The virulence gene activator ToxT from *Vibrio cholerae* is a

© 2000 Blackwell Science Ltd, *Molecular Microbiology*, **38**, 67–84

member of the AraC family of transcriptional activators. *J Bacteriol* **174**: 6974–6980.

Higuchi, R. (1990) Recombinant PCR. In *PCR Protocols: a Guide to Methods and Applications.* Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J. (eds). San Diego: Academic Press, p. 177–183.

Ishihama, A. (1992) Role of RNA polymerase a subunit in transcription activation. *Mol Microbiol* **6**: 3283–3288.

Karaolis, D.K., Johnson, J.A., Bailey, C.C., Boedeker, E.C., Kaper, J.B., and Reeves, P.R. (1998) A *Vibrio cholerae* pathogenicity island associated with epidemic and pandemic strains. *Proc Natl Acad Sci USA* **95**: 3134–3139.

Karaolis, D.K.R., Somara, S., Manaeval, D.R., Jr, Johnson, J.A., and Kaper, J.B. (1999) A bacteriophage encoding a pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria. *Nature* **399**: 375–379.

Kovach, M.E., Shaffer, M.D., and Peterson, K.M. (1996) A putative integrase gene defines the distal end of a large cluster of ToxR-regulated colonization genes in *Vibrio cholerae*. *Microbiology* **142**: 2165–2174.

Kovacikova, G., and Skorupski, K. (1999) A *Vibrio cholerae* LysR homolog, AphB, cooperates with AphA at the *tcpPH* promoter to activate expression of the ToxR virulence cascade. *J Bacteriol* **181**: 4250–4256.

Li, C.C., Crawford, J.A., DiRita, V.J., and Kaper, J.B. (2000) Molecular cloning and transcriptional regulation of *ompT*, a ToxR-repressed gene in *Vibrio cholerae*. *Mol Microbiol* **35**: 189–203.

Makino, K., Amemura, M., Kawamoto, T., Kimura, S., Shinagawa, H., Nabata, A., *et al.* (1996) DNA binding of PhoB and its interaction with RNA polymerase. *J Mol Biol* **259**: 15–26.

Martínez-Hackert, E., and Stock, A.M. (1997a) Structural relationships in the OmpR family of winged-helix transcription factors. *J Mol Biol* **269**: 301–312.

Martínez-Hackert, E., and Stock, A.M. (1997b) The DNA-binding domain of OmpR: crystal structure of a winged helix transcription factor. *Structure* **5**: 109–124.

Miller, J.H. (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Miller, V.L., and Mekalanos, J.J. (1988) A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J Bacteriol* **170**: 2575–2583.

Miller, V.L., Taylor, R.K., and Mekalanos, J.J. (1987) Cholera toxin transcriptional activator ToxR is a transmembrane DNA binding protein. *Cell* **48**: 271–279.

Murley, Y.M., Carroll, P.A., Skorupski, K., Taylor, R.K., and Calderwood, S.B. (1999) Differential transcription of the *tcpPH* operon confers biotype-specific control of the *Vibrio cholerae* ToxR virulence regulon. *Infect Immun* **67**: 5117–5123.

Ogierman, M.A., Voss, E., Meaney, C., Faast, R., Attridge, S.R., and Manning, P.A. (1996) Comparison of the promoter proximal regions of the toxin-co-regulated *tcp* gene cluster in classical and El Tor strains of *Vibrio cholerae* O1. *Gene* **170**: 9–16.

Ottemann, K.M., DiRita, V.J., and Mekalanos, J.J. (1992) ToxR proteins with substitutions in residues conserved with OmpR fail to activate transcription from the cholera toxin promoter. *J Bacteriol* **174**: 6807–6814.

Peterson, K.M., and Mekalanos, J.J. (1988) Characterization of the *Vibrio cholerae* ToxR regulon: identification of novel genes involved in intestinal colonization. *Infect Immun* **56**: 2822–2829.

Pratt, L.A., and Silhavy, T.J. (1994) OmpR mutants specifically defective for transcriptional activation. *J Mol Biol* **243**: 579–594.

Rhodius, V.A., and Busby, S.J.W. (1998) Positive activation of gene expression. *Curr Opin Microbiol* **1**: 152–159.

Russo, F.D., Slauch, J.M., and Silhavy, T.J. (1993) Mutations that affect separate functions of OmpR the phosphorylated regulator of porin transcription in *Escherichia coli*. *J Mol Biol* **231**: 261–273.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Skorupski, K., and Taylor, R.K. (1996) Broad-host-range positive selection vectors for allelic exchange. *Gene* **169**: 47–52.

Skorupski, K., and Taylor, R.K. (1999) A new level in the *Vibrio cholerae* virulence cascade: aphA is required for transcriptional activation of the *tcpPH* operon. *Mol Microbiol* **31**: 763–771.

Svennerholm, A.M., and Holmgren, J. (1978) Identification of the *Escherichia coli* heat-labile enterotoxin by means of a ganglioside immunosorbent assay (GM_1-ELISA) procedure. *Curr Microbiol* **1**: 19–23.

Taylor, R.K., Miller, V.L., Furlong, D.B., and Mekalanos, J.J. (1987) Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. *Proc Natl Acad Sci USA* **84**: 2833–2837.

Thomas, S., Williams, S.G., and Manning, P.A. (1995) Regulation of tcp genes in classical and El Tor strains of *Vibrio cholerae* O1. *Gene* **166**: 43–48.

Waldor, M.K., and Mekalanos, J.J. (1996) Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272**: 1910–1914.

Wong, S.M., Carroll, P.A., Rahme, L.G., Ausubel, F.M., and Calderwood, S.B. (1998) Modulation of expression of the ToxR regulon in *Vibrio cholerae* by a member of the two-component family of response regulators. *Infect Immun* **66**: 5854–5861.

Yu, R.R., and DiRita, V.J. (1999) Analysis of an autoregulatory loop controlling ToxT, cholera toxin, and toxin-coregulated pilus production in *Vibrio cholerae*. *J Bacteriol* **181**: 2584–2592.