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The two faces of ToxR: activator of *ompU*, co-regulator of *toxT* in *Vibrio cholerae*

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Summary

ToxR of *Vibrio cholerae* directly activates the *ompU* promoter, but requires a second activator, TcpP to activate the *toxT* promoter. *ompU* encodes a porin, while *toxT* encodes the transcription factor, ToxT, which activates *V. cholerae* virulence genes including cholera toxin and the toxin co-regulated pilus. Using an *ompU-sacB* transcriptional fusion, *toxR* mutant alleles were identified that encode ToxR molecules defective for *ompU* promoter activation. Many *toxR* mutants defective for *ompU* activation affected residues involved in DNA binding. Mutants defective for *ompU* activation were also tested for activation of the *toxT* promoter. ToxR-F69A and ToxR-V71A, both in the α-loop of ToxR, were preferentially defective for *ompU* activation, with ToxR-V71A nearly completely defective. Six mutants from the *ompU-sacB* selection showed more dramatic defects in *toxT* activation than *ompU* activation. All but one of the affected residues map to the wing domain of the winged helix–turn–helix of ToxR. Some ToxR mutants preferentially affecting *toxT* activation had partial DNA-binding defects, and one mutant, ToxR-P101L, had altered interactions with TcpP. These data suggest that while certain residues in the α-loop of ToxR are utilized to activate the *ompU* promoter, the wing domain of ToxR contributes to both promoter binding and ToxR/TcpP interaction facilitating *toxT* activation.

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Introduction

Vibrio cholerae is the causative agent of the diarrheal disease cholera, a disease estimated to affect 18 million people each year worldwide (WHO, 2003). *V. cholerae* lives in aquatic environments year-round, but during seasonal outbreaks the bacterium is ingested by humans in contaminated food or water. Following ingestion, *V. cholerae* senses the changes in environmental conditions and responds by activating a number of genes whose products are required for effective colonization and pathogenesis in human hosts. These include the genes encoding cholera toxin and the toxin co-regulated pilus among others (Mekalanos *et al.*, 1983; Taylor *et al.*, 1987; Levine *et al.*, 1988; Peterson and Mekalanos, 1988). Activation of these virulence genes is dependent upon two inner membrane localized transcription factors ToxR and TcpP (Miller and Mekalanos, 1984; Miller *et al.*, 1987; Carroll *et al.*, 1997; Häse and Mekalanos, 1998). Both ToxR and TcpP have C-terminal periplasmic domains with the potential to sense environmental signals as well as N-terminal DNA binding and transcription activation domains similar to the winged helix–turn–helix (winged-HTH) family of transcription factors (Martinez-Hackert and Stock, 1997). ToxR and TcpP stimulate virulence gene expression by combining to activate the *toxT* promoter (Häse and Mekalanos, 1998; Murley *et al.*, 1999; Krukonis *et al.*, 2000). ToxT then activates various virulence genes directly (DiRita *et al.*, 1991; Yu and DiRita, 2002; Withey and DiRita, 2006).

DNAse I footprinting studies, *toxT* promoter mutations and analysis of a series of *tcpP* mutants have suggested that TcpP directly interacts with RNA polymerase at the *toxT* promoter while ToxR serves an accessory role from a position further upstream of the basal promoter elements (Krukonis *et al.*, 2000; Krukonis and DiRita, 2003; Goss *et al.*, 2010). Furthermore, it has been demonstrated that TcpP and ToxR physically interact and that this interaction may contribute to the activity of ToxR on the *toxT* promoter (Krukonis and DiRita, 2003). In fact, a mutant derivative of TcpP that is deficient in *toxT* promoter binding (TcpP-H93L) is still functional for *toxT* activation if ToxR is coexpressed (Krukonis *et al.*, 2000). TcpP binds a pentameric direct repeat element TGTAA-N_6-TGTAA from –53 to –38 relative to the start site of *toxT* transcription (Goss *et al.*, 2010). Within this repeat element, the central

nucleotide of both repeats is critical for TcpP-mediated *toxT* activation, even in the presence of ToxR (Goss *et al*., 2010). These data lead to a model in which ToxR binds a region upstream of the TcpP-binding site and facilitates *toxT* promoter recognition and activation by TcpP (Goss *et al*., 2010).

While both ToxR and TcpP are required for *toxT* activation (Higgins and DiRita, 1994; Champion *et al*., 1997; Häse and Mekalanos, 1998), ToxR is able to regulate other promoters in a TcpP-independent fashion. One such promoter, *ompU*, controls expression of the outer membrane porin OmpU. ToxR directly activates *ompU* (Crawford *et al*., 1998) and represses expression of the alternative porin, OmpT (Li *et al*., 2000). Proper regulation of outer membrane protein synthesis is critical as expression of OmpT leads to greater sensitivity to bile and other related detergents (Provenzano and Klose, 2000).

Given that ToxR directly activates *ompU* yet plays an accessory role in *toxT* activation, we hypothesized that the molecular mechanism by which ToxR regulates these two promoters may be different and we might be able to isolate point mutations in *toxR* that affect activation of *ompU* or *toxT* specifically. Identification of such mutants would strengthen the hypothesis that different regions of ToxR are more critical for one type of activation or the other, and based on homology to the winged-HTH family of transcription factors, the position of mutant substitutions may direct us to the function of certain domains of ToxR. Precedent for such differential activation of the *ompU* and *toxT* promoters comes from the fact that *toxT* expression requires that ToxR be membrane-localized, while *ompU* can be efficiently activated by a soluble form of ToxR (Crawford *et al*., 2003).

In this study we identified residues in the α-loop of ToxR, especially V71, as being critical for *ompU* promoter activation, while several ToxR mutants had preferential defects in *toxT* promoter activation, including six in the wing domain of ToxR.

Results

We hypothesized that as ToxR appears to function by different mechanisms at the *ompU* and *toxT* promoters, we might be able to isolate *toxR* mutants that affect *ompU* transcription, but not *toxT*. Identification of such mutants would indicate which domain(s) of ToxR is critical to directly activate transcription at the *ompU* promoter.

Isolation of toxR *alleles defective for* ompU *activation*

To isolate *toxR* mutants defective for *ompU* activation, an *ompU-sacB* transcriptional fusion strain was constructed in which an *ompU-sacB* fusion was inserted at the *lacZ* locus of the Δ*toxR* classical *V. cholerae* strain EK307 (EK406 derived from EK307; Table 1). The Δ*toxR ompU-sacB* selection strain is able to grow on 5% sucrose; however, upon introduction of a wild-type copy of *toxR* on a plasmid, this strain becomes sensitive to 5% sucrose. To isolate *toxR* mutants, three pools of mutagenized *toxR* alleles were introduced into EK406 by electroporation. Transformants were plated on Luria–Bertani (LB) plates containing 5% sucrose and colonies capable of growing on 5% sucrose were isolated. The *toxR* encoding plasmids were sequenced, and selected mutants were studied further.

In the course of constructing the *toxR* allele for PCR mutagenesis, a C-terminal (periplasmic) HA-epitope tag was added to the *toxR* coding sequence to allow assessment of protein stability and translation with an anti-HA tag antibody (*Experimental procedures*).

Quantification of ompU *activation defects*

Once candidate sucrose-resistant *toxR* alleles were identified that were defective for *ompU* activation, their protein stability was assessed by anti-HA-epitope tag Western blot analysis (data not shown) and alleles encoding full-length ToxR derivatives were introduced into a *V. cholerae* Δ*toxR ompU-lacZ* reporter strain (EK410) to quantify *ompU* activation defects. Like the *ompU-sacB* selection strain the *ompU-lacZ* reporter was inserted at the *lacZ* locus of *V. cholerae* (Crawford *et al*., 2003). The various plasmid-encoded ToxR derivatives were tested for their ability to activate *ompU* as compared with wild-type ToxR or an empty vector control (pSK). Only mutants harbouring a single amino acid substitution were studied further. If a sequenced *toxR* allele defective for *ompU* activation contained two or more mutations, the mutations were separated by allelic exchange of a restriction fragment into the parental plasmid pSK-*toxR*-HA or by site-directed mutation. Following these reconstructions, 13 mutants from the randomly mutagenized pools were studied. β-galactosidase assays were performed and Miller Units for wild-type ToxR were set at 100% and all mutant derivatives were expressed relative to 100%.

Of these 13 ToxR derivatives, seven showed little or no ability to activate the *ompU* promoter (≤ 3% wild-type activity); ToxR-W64R, ToxR-V71A, ToxR-Q78R, ToxR-L83P, ToxR-T99K, ToxR-T99R and ToxR-G104S, although ToxR-L83P was somewhat less stable than the other mutants (Fig. 1). The remaining six mutants retained 24–79% activity relative to wild-type ToxR.

Effect of ToxR substitutions on toxT *activation*

While the ToxR mutants were isolated based on their reduced activation of the *ompU* promoter, we were also interested in whether any mutants showed a differential

Table 1. Strains and plasmids.

	Source
V. cholerae strain	
EK307 O395 Δ*toxR*	Krukonis *et al.* (2000)
EK406 EK307 *ompU-sacB*	This study
EK410 EK307 *ompU-lacZ*	Crawford *et al.* (2003)
EK459 O395 Δ*toxR*Δ*tcpP*	Krukonis *et al.* (2000)
EK816 O395 Δ*toxRS toxT-lacZ*	This study
EK1072 EK307 *toxT-lacZ*	This study
E. coli strain	
DH5α	lab strain
DHM1	Karimova *et al.* (2005)
BTH101	Karimova *et al.* (2001)
Plasmids	
Random mutants from *ompU-sacB*	
pSK-*toxR-HA* wild-type	This study
pSK-*toxR-HA*-W64R	This study
pSK-*toxR-HA*-R65Q	This study
pSK-*toxR-HA*-V71A	This study
pSK-*toxR-HA*-Q78R	This study
pSK-*toxR-HA*-L83P	This study
pSK-*toxR-HA*-D89E	This study
pSK-*toxR-HA*-K92E	This study
pSK-*toxR-HA*-S93P	This study
pSK-*toxR-HA*-T99K	This study
pSK-*toxR-HA*-T99R	This study
pSK-*toxR-HA*-P101L	This study
pSK-*toxR-HA*-R103G	This study
pSK-*toxR-HA*-G104S	This study
pSK-*toxR-HA*-L107S	This study
α-loop mutants	
pSK-*toxR-HA*-R65A	This study
pSK-*toxR-HA*-E66A	This study
pSK-*toxR-HA*-Q67A	This study
pSK-*toxR-HA*-G68A	This study
pSK-*toxR-HA*-F69A	This study
pSK-*toxR-HA*-E70A	This study
pSK-*toxR-HA*-D72A	This study
pSK-*toxR-HA*-D73A	This study
pSK-*toxR-HA*-S74A	This study
pSK-*toxR-HA*-S75A	This study
Ottemann reconstructed mutants	Ottemann *et al.* (1992)
pSK-*toxR-HA*-E39K (formerly E51K)	This study
pSK-*toxR-HA*-R56K	This study
pSK-*toxR-HA*-R56L	This study
pSK-*toxR-HA*-R65L	This study
pSK-*toxR-HA*-R84K	This study
pSK-*toxR-HA*-R84L	This study
DiRita ToxS-blind mutants	DiRita and Mekalanos (1991)
pSK-*toxR-HA*-K85E	This study
pSK-*toxR-HA*-D89N	This study
pSK-*toxR-HA*-T99M	This study
Random mutants (*toxT-lacZ*)	
pMMB66EH-*toxR*	This study
pSK-*toxR-HA*-R84C	This study
pSK-*toxR-HA*-D89Y	This study
pSK-*toxR-HA*-K98E	This study
Bacterial two-hybrid plasmids	
pKT25	Karimova *et al.* (2001)
pUT18c	Karimova *et al.* (2001)
pUT18c-EpsM	This study
pUT18c-ToxRS	This study
pUT18c-ToxRS-D73A	This study
pUT18c-ToxRS-D89E	This study
pUT18c-ToxRS-K92E	This study
pUT18c-ToxRS-S93P	This study
pUT18c-ToxRS-K98E	This study
pUT18c-ToxRS-P101L	This study
pUT18c-ToxRS-R103G	This study
pKT25-TcpPH	This study
pKT25-TcpPH-L96S	This study
pKT25-TcpPH-I97T	This study
pKT25-TcpPH-K101E	This study

effect on *toxT* activation. If the mechanism of *ompU* and *toxT* activation differs, we expected to find some mutants that were defective for *ompU* activation to be less affected for *toxT* activation.

To examine the effect of ToxR substitutions on *toxT* activation, the various plasmid-borne alleles were introduced into the Δ*toxR toxT-lacZ* reporter strain EK1072 (Table 1). Of the seven ToxR mutants that showed little or no *ompU-lacZ* expression, all but mutant ToxR-V71A showed little or no activity for *toxT-lacZ* expression as well (Fig. 1). While many of these mutants may be affected in their DNA-binding activity (see below), ToxR-V71A seemed to be particularly interesting as it showed only 3% activity on the *ompU* promoter, but maintained 15% activation of the *toxT* promoter (Fig. 1). This suggests the ToxR-V71A mutation affects a region of ToxR that is especially important for ToxR to directly activate the *ompU* promoter, but less critical when ToxR plays an accessory role with TcpP to activate the *toxT* promoter. Based on homology with other winged-HTH proteins, residue V71 of ToxR lies within the α-loop that is proposed to directly interact with RNA polymerase to activate transcription of target promoters (Fig. 2A and B). Thus, some mutations in this region of ToxR would be predicted to affect the direct activation mechanism of ToxR at the *ompU* promoter more than the accessory role at the *toxT* promoter.

In addition to identifying mutant ToxR-V71A that affects *ompU* activation more dramatically than *toxT*, six of the ToxR mutants isolated were more strongly attenuated for *toxT* activation than *ompU*. While this phenotype was not necessarily expected from our selection strategy, we were excited to identify such differential ToxR mutants. Again, such mutants suggest that activation of *ompU* and *toxT* by ToxR have different specific requirements within the transcription activator ToxR. Five of six mutants lie just N-terminal to or within the putative wing domain of the ToxR winged-HTH structure; ToxR-D89E, ToxR-K92E, ToxR-S93P, ToxR-P101L and ToxR-R103G (Fig. 2A and C). The wing domain of winged-HTH proteins can perform different functions including DNA binding and protein–protein interaction, depending on the activator protein (Littlefield and Nelson, 1999; Gajiwala *et al.*, 2000; Blanco *et al.*, 2002; Krukonis and DiRita, 2003).

When the ToxR sequence is threaded onto the structures of related winged-HTH activator proteins (Fig. 2A), five of the amino acid substitutions preferentially affecting *toxT* expression are predicted to lie on a distinct face of ToxR (Fig. 2C) from amino acids required for efficient *ompU* activation in the α-loop (Fig. 2B). We did identify one α-loop mutant, ToxR-R65Q, in the *ompU-sacB* screen that was preferentially defective for *toxT* activation, but it was the least preferential (< twofold differential, Figs 1 and 2C).

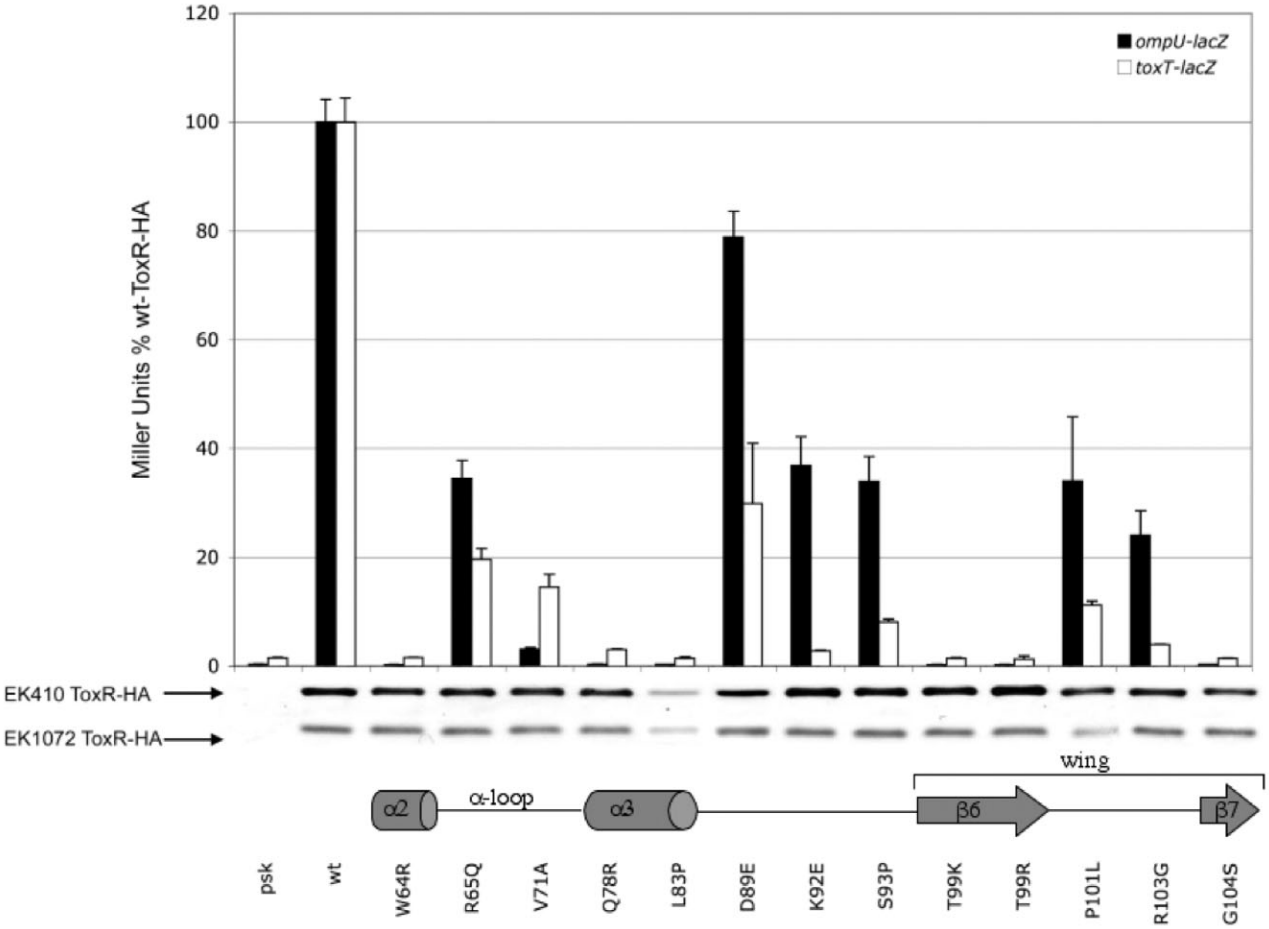

Fig. 1. Activation of *ompU-lacZ* or *toxT-lacZ* chromosomal reporter constructs in *V. cholerae* by various ToxR mutant derivatives. *ΔtoxR V. cholerae* strains were complemented with HA-tagged wild-type ToxR, the empty vector pSK Bluescript, or various ToxR mutant proteins. β-galactosidase activity was measured after a 3–4 h induction with 100 μM IPTG at 30°C. Miller units were expressed as percent, relative to activation seen with wild-type ToxR-HA. Data shown are from at least two experiments performed in triplicate. ToxR-HA from the same samples used in the β-galactosidase assay was detected with an anti-HA antibody to assess protein stability.

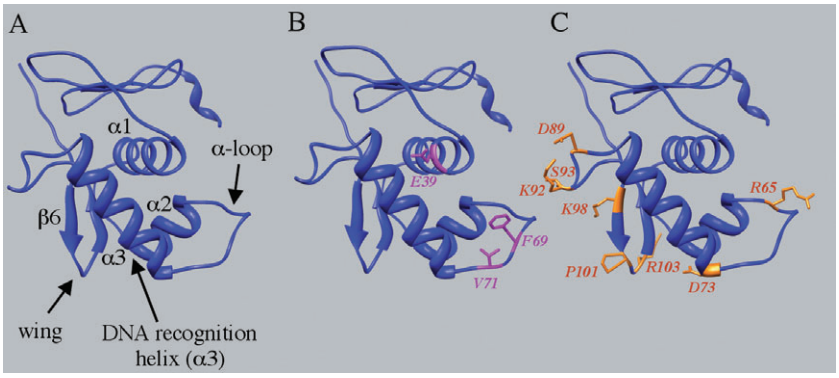

Fig. 2. Modelling of the N-terminal DNA-binding/transactivation domain of ToxR (residues 1–114) using threading of the ToxR sequence onto structurally related winged-HTH family members.
A. Labelling of putative domains of interest within the ToxR transcription activation and DNA-binding domain, model assembled using I-TASSER (Zhang, 2008; 2009; Roy *et al*., 2010).
B. Homology modelling of residues within ToxR that affect *ompU* promoter activation more dramatically than *toxT* promoter activation.
C. Homology modelling of residues within ToxR that affect *toxT* promoter activation more dramatically than *ompU* promoter activation.

A preliminary screen for ToxR mutants defective for *toxT-lacZ* activation identified one additional mutant, ToxR-K98E, preferentially affecting *toxT* activation (26% of wild type) more than *ompU* activation (66% of wild type, Table S1). This mutant was isolated using a blue/white plate screen for *toxT-lacZ* activation (*Supporting information*). ToxR-K98 also lies in the putative wing domain of ToxR like most other *toxT* preferentially defective ToxR mutants.

Finally, a number of activation-defective ToxR mutants have been identified previously. We assessed nine such mutants (DiRita and Mekalanos, 1991; Ottemann *et al*., 1992) for *toxT* and *ompU* activation (Table S1) and DNA binding (Fig. S1). One mutant, ToxR-E39K, had a differential effect on activation. ToxR-E39K activated the *ompU* promoter to 19% of wild-type ToxR, while it activated the *toxT* promoter at 105% of wild-type levels (Table S1). This residue is thought to play a structural role in domain packing of the winged-HTH domain of PhoB/OmpR family members (Martínez-Hackert and Stock, 1997; Okamura *et al*., 2000; Blanco *et al*., 2002), but may also influence ToxR/RNAP interaction at the *ompU* promoter (Fig. 2B).

DNA-binding activity of ToxR mutant derivatives

Six of the 13 isolated ToxR mutants were severely defective for activation of both the *ompU* and *toxT* promoters. These include ToxR-W64R, ToxR-Q78R, ToxR-L83P, ToxR-T99K, ToxR-T99R and ToxR-G104S. One simple explanation for the defect in activation by these mutants is that they fail to recognize either promoter. In fact, two of the seven mutants lie within the putative DNA-recognition helix (α3, Figs 1 and 2A) of ToxR (ToxR-Q78R and ToxR-L83P).

We first assessed binding to the *ompU* promoter. *V. cholerae* lacking endogenous copies of *toxR* and *tcpP* (strain EK459, Table 1) was transformed with the pSK-*toxR-HA* plasmid encoding each *toxR* mutant allele. Membranes were prepared from these strains (Miller *et al*., 1987) and used in a gel mobility shift assay with a radiolabelled fragment of the *ompU* promoter from –211 to +22 relative to the transcription start site. This fragment has been shown previously to contain ToxR-binding sites (Crawford *et al*., 1998).

ompU promoter probes were mixed with 0.05 or 0.25 mg ml^{-1} of total membrane proteins. While negative control membranes (pSK, vector alone) gave some shifting of the probe in the absence of ToxR (Fig. 3A, asterisk), ToxR-mediated binding could be distinguished because ToxR causes retention of the probe in the well (membranes are retained in the well in this assay, Fig. 3A). This experiment demonstrated that the six ToxR derivatives severely defective for both *ompU* and *toxT* activation were unable to bind the *ompU* promoter. These include ToxR-W64R, ToxR-Q78R, ToxR-L83P, ToxR-T99K, ToxR-T99R and ToxR-G104S. To confirm that sufficient amounts of ToxR were available to bind the *ompU* promoter, Western blot analysis was performed to determine the relative amount of each ToxR derivative present in each gel-shift reaction. Levels of all 14 ToxR proteins (including wild type) were similar (Fig. 3A). As a negative control probe for these experiments we used a fragment of the *toxT* promoter lacking a ToxR-binding site (from –46 to +45).

Most ToxR derivatives that demonstrated an intermediate *ompU* activation defect (Fig. 1) showed either modest or no DNA-binding defect for the *ompU* promoter (Fig. 3A). While ToxR-R65Q, ToxR-V71A and ToxR-K92E were clearly not as efficient as wild-type ToxR for *ompU* promoter binding (even with ToxR-K92E being present at higher protein levels than wild-type ToxR), ToxR-P101L and ToxR-R103G were somewhat less obvious in their DNA-binding defects. Finally, ToxR-D89E and ToxR-S93P bind the *ompU* promoter similar wild-type ToxR, although ToxR-S93P does not retain all of the *ompU* probe in the well (some smearing).

Because the ToxR derivatives with intermediate *ompU* activation defects all have differential effects on *toxT* activation (Fig. 1), we examined whether these ToxR derivatives have differential defects in *toxT* promoter binding as compared with the *ompU* promoter. A *toxT* promoter probe (from –172 to +45) containing the ToxR-binding site was radiolabelled and used in a gel-shift assay with the same membranes used for the *ompU* promoter gel-shift assays (Fig. 3B). The six mutants defective for both *ompU* and *toxT* activation (ToxR-W64R, ToxR-Q78R, ToxR-L83P, ToxR-T99K, ToxR-T99R and ToxR-G104S) were also defective for *toxT* promoter binding.

The two α-loop mutants, ToxR-R65Q and ToxR-V71A showed intermediate defects in *toxT* promoter binding, consistent with their ability to activate the *toxT* promoter to 15–20% of wild-type ToxR activity (Fig. 1).

The five ToxR mutants in the wing domain (or just N-terminal to the wing) had intermediate *toxT* promoter-binding defects (Fig. 3B). These wing and wing-proximal mutants appear slightly more defective for *toxT* than *ompU* promoter binding (Fig. 3A vs. Fig. 3B). However, the level of their *toxT* activation defect is not solely determined by their DNA-binding defect. Specifically, ToxR-P101L and ToxR-R103G have similar *toxT* promoter DNA-binding defects, yet ToxR-R103G is threefold more defective for *toxT* activation than ToxR-P101L. Thus, this region may perform a role in addition to DNA binding that affects *toxT* transcription activation. While ToxR-K92E maintains some (albeit modest) DNA-binding activity on the *ompU* and *toxT* promoters (Fig. 3), it has a much stronger defect in *toxT* activation than *ompU* activation

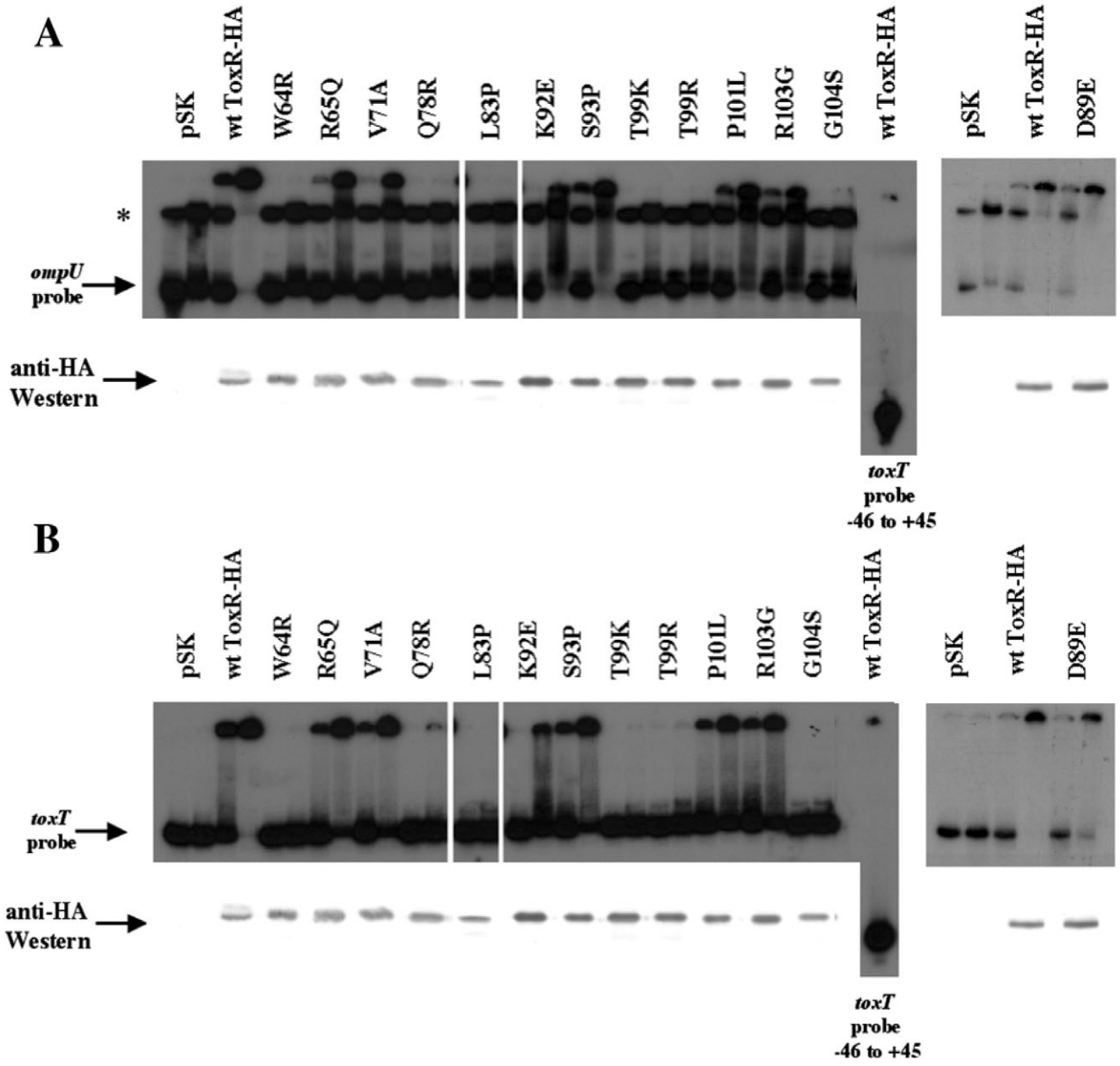

Fig. 3. Gel-shift assays to assess promoter recognition by various ToxR mutant proteins identified in a random mutagenesis *ompU-sacB* selection strategy. *V. cholerae* membranes prepared from a *ΔtoxRΔtcpP V. cholerae* strain (EK459) expressing each mutant ToxR derivative (or wild-type ToxR-HA) were mixed at 0.05 and 0.25 mg ml^{-1} (two lanes for each sample) with radiolabelled *ompU* promoter DNA (A) or 0.1 and 0.5 mg ml^{-1} with *toxT* promoter DNA (B) before running samples in a non-denaturing PAGE.

(Fig. 1). This could reflect a critical role for residue K92 in *toxT* activation or the fact that *toxT* promoter activation is more sensitive to ToxR mutations affecting DNA-binding affinity. Finally, mutant ToxR-D89E showed nearly wild-type binding to both the *ompU* and *toxT* promoters, with a very slight defect in *toxT* binding (Fig. 3). This mutant can only activate the *toxT* promoter to about 30% the level of wild-type ToxR, while *ompU* activation shows 79% of wild-type activity. This suggests this conservative amino acid substitution is particularly detrimental to ToxR/TcpP-mediated activation of the *toxT* promoter because of either a slight defect in *toxT* promoter binding or some other *toxT* activation function.

The role of α-loop residues on ompU *and* toxT *activation by ToxR*

Because we found one ToxR residue essential for *ompU* expression in the α-loop of ToxR, ToxR-V71 (Fig. 1), we performed alanine-scanning mutagenesis of the entire the

Table 2. Activation phenotypes of ToxR α-loop mutants.

ToxR derivative	location	*ompU-lacZ*	*toxT-lacZ*
ToxR-HA wild type	–	100.0% ± 5.6	100.0% ± 6.9
pSK (vector)	–	0.3% ± 0.03	1.6% ± 0.1
ToxR-R65A	α-loop	51.3% ± 2.9	43.1% ± 1.4
ToxR-E66A	α-loop	103.3% ± 4.6	106.4% ± 2.0
ToxR-Q67A	α-loop	101.4% ± 3.6	164.5% ± 3.9
ToxR-G68A	α-loop	12.0% ± 0.9	19.2% ± 2.3
ToxR-F69A	α-loop	35.2% ± 1.1	91.6% ± 3.0
ToxR-E70A	α-loop	79.5% ± 3.4	91.7% ± 3.1
ToxR-D72A	α-loop	102.8% ± 2.5	118.0% ± 9.5
ToxR-D73A	α-loop	92.9% ± 0.6	40.8% ± 15.3
ToxR-S74A	α-loop	47.4% ± 5.1	63.1% ± 6.7
ToxR-S75A	α-loop/DNA- binding domain	0.4% ± 0.02	4.5% ± 0.3

Miller units measured in strains EK410 (O395 Δ*toxR ompU-lacZ*) and EK1072 (O395 Δ*toxR toxT-lacZ*). Data are from one representative experiment performed in triplicate.

putative α-loop (R65-S75). Mutations were constructed in the pSK-*toxR-HA* vector and expressed in the either the *ompU-lacZ* (EK410) or *toxT-lacZ* (EK1072) reporter strains to assess transcription activation activity.

One additional mutant ToxR protein, ToxR-F69A, showed a > twofold preferential defect for *ompU* expression. It activated *ompU-lacZ* to 35% of wild-type levels and *toxT-lacZ* to 92% of wild type (Table 2). Three mutants, ToxR-R65A, ToxR-G68A and ToxR-S74A, showed decreases in both *ompU* and *toxT* expression, ToxR-G68A and ToxR-S74A being slightly more defective for *ompU* than *toxT* activation (Table 2). One mutant protein, ToxR-S75A, showed little or no activation of either *ompU* or *toxT*. This residue approaches the predicted DNA-binding helix of ToxR and disrupts the DNA-binding activity of ToxR (see below).

One alanine mutant in the α-loop, ToxR-D73A, showed a more dramatic defect on *toxT* (41% of wild type) than on *ompU* (93% of wild type) expression. When residue D73 of ToxR is modelled onto homologous winged-HTH transcription factor structures, ToxR residues F69 and V71 are oriented away from the α-loop in one direction, while residue D73 is predicted to be oriented in the opposite direction, towards residues P101 and R103 (Fig. 2C). Our random mutagenesis selection identified mutations in these latter two residues, ToxR-P101L and ToxR-R103G, which are also more dramatically affected for *toxT* expression than *ompU*. Thus, this face of the ToxR molecule appears to play a critical role in *toxT* activation in conjunction with TcpP, while V71 and F69 of the α-loop are more important for *ompU* activation by ToxR.

These alanine mutant studies also revealed that the preferential defect of ToxR-R65Q on *toxT* activation (Fig. 1) was largely side-chain-dependent as the ToxR-R65A mutant had more similar defects in both *ompU* and *toxT* activation (albeit still slightly more defective for *toxT* activation, Table 2).

Promoter recognition by α-loop alanine mutants

Because some of our α-loop ToxR alanine mutants have activation defects, we assessed the DNA-binding activity of the various mutant proteins. Membranes were isolated from a *V. cholerae ΔtoxRΔtcpP* mutant (EK459) expressing various ToxR derivatives and the membrane preparations were used in gel-shift assays on the *ompU* (Fig. 4A) or *toxT* (Fig. 4B) promoter.

While wild-type ToxR shifted about 50–75% of the *ompU* probe at the lower membrane concentration (Fig. 4A), three mutant proteins partially defective for *ompU* activation (ToxR-R65A, ToxR-G68A and ToxR-S74A; Table 2) had somewhat weaker binding to the *ompU* promoter. ToxR-S75A is unable to activate either the *ompU* or *toxT* promoter (Table 2) and is severely defective for DNA binding (Fig. 4). Thus, it may define the first residue of the DNA-binding helix of ToxR. Finally, ToxR-F69A maintains wild-type levels of binding to the *ompU* promoter (Fig. 4A) and displays slightly reduced binding to the *toxT* promoter (Fig. 4B). However, ToxR-F69A is preferentially defective for *ompU* activation (Table 2). Thus, residue F69 (like V71 identified in our original random mutagenesis selection) appears to be particularly important for ToxR to directly activate RNAP at the *ompU* promoter.

Effect of specific ToxR wing residue substitutions on differential promoter activation

Because alanine-scanning mutagenesis of the α-loop indicated some preferential defects were side-chain-dependent (R65Q vs. R65A, Fig. 1 and Table 2), we determined whether the preferential *toxT* activation defects of certain ToxR wing mutants were due to the specific side-chain substitutions, each residue was mutated to alanine (ToxR-D89A, ToxR-K92A, ToxR-S93A,

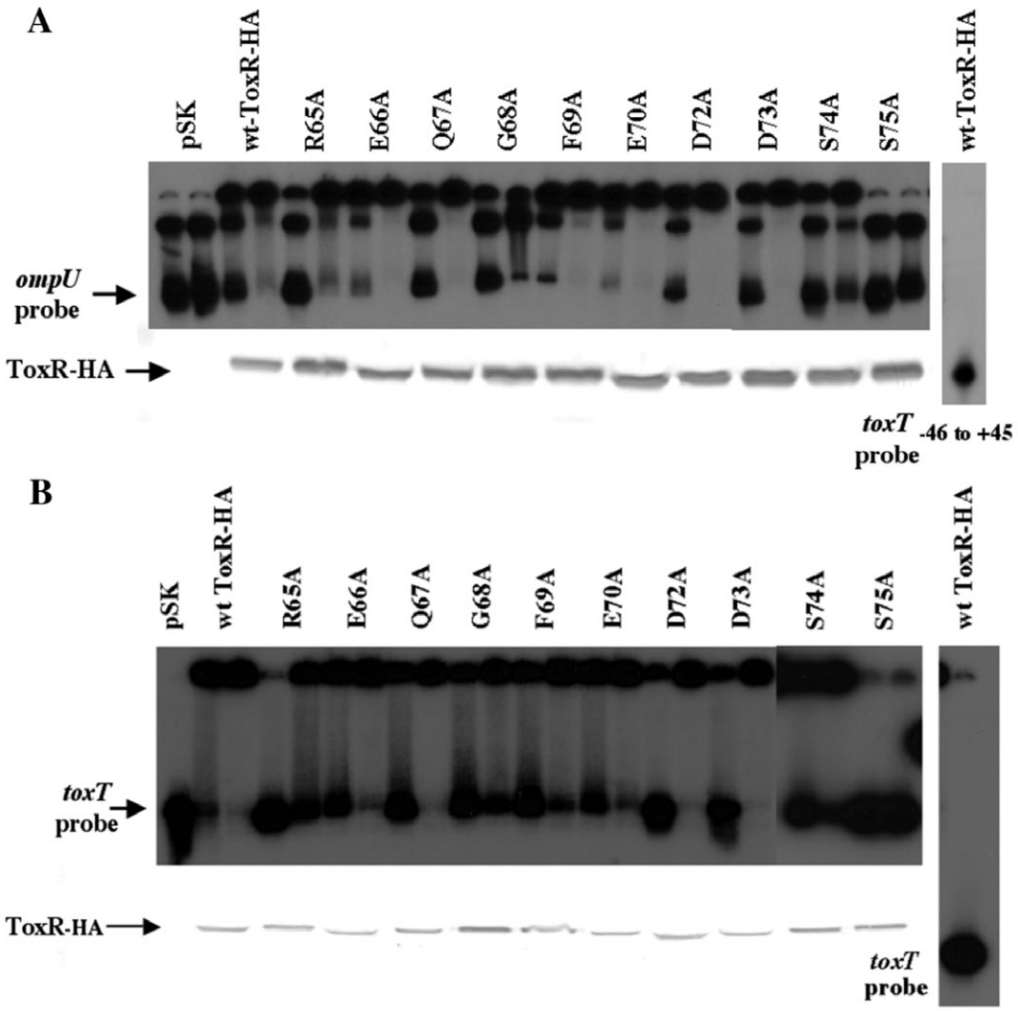

Fig. 4. Gel-shift assays to assess promoter recognition by putative α-loop site-directed ToxR mutant proteins. *V. cholerae* membranes prepared from a *ΔtoxRΔtcpP V. cholerae* strain (EK459) expressing each mutant ToxR derivative (or wild-type ToxR-HA) were mixed at 0.05 and 0.25 mg ml^{-1} with radiolabelled *ompU* promoter DNA (A) or 0.2 and 1.0 mg ml^{-1} with *toxT* promoter DNA (B) before running samples in a non-denaturing PAGE.

ToxR-K98A, ToxR-P101A and ToxR-R103A). These ToxR derivatives were then tested for *ompU* and *toxT* activation in *V. cholerae*. While the absolute levels of *ompU* and *toxT* activation changed in the alanine mutants relative to the original side-chain substitution mutants, five of six wing mutants tested maintained preferential defects in *toxT* activation; ToxR-K92A, ToxR-S93A ToxR-K98A, ToxR-P101A and ToxR-R103A (Table 3), indicating these *toxT*-specific defects were not side-chain-dependent.

The ToxR-D89A mutant was completely defective for both *ompU* and *toxT* activation (Table 3) whereas the original conservative mutant, ToxR-D89E, maintained some activation of *ompU* and *toxT* (although it was preferentially *toxT*-defective, Fig. 1). All six alanine wing mutants were stably expressed similar to wild-type ToxR (data not shown).

Mutant ToxR-P101L in the putative wing domain of ToxR is deficient in ToxR-TcpP cross-linking

Given that ToxR appears to play a supporting role for activation of the *toxT* promoter by assisting direct activation by TcpP (Krukonis and DiRita, 2003), we tested our various ToxR wing domain mutants that were preferentially affected for *toxT-lacZ* activation, for their ability to interact with TcpP in the membrane of *V. cholerae*. We used a cross-linking capture ELISA protocol described previously (Krukonis and DiRita, 2003) for assessing the interaction of a number of ToxR mutant derivatives with wild-type TcpP. For these experiments a *V. cholerae ΔtcpP* strain (RY1) was first modified to harbour each *toxR* allele of interest on the chromosome at the *toxR* locus. The resulting strains were transformed with an HSV

Table 3. Affect of side-chain substitutions in the wing domain on promoter activation.

ToxR derivative	*ompU-lacZ* (% wild type)	*toxT-lacZ* (% wild type)
ToxR-HA wild type	100 ± 3.5	100 ± 5.0
pSK (vector)	0.30 ± 0.04	1.4 ± 0.07
ToxR-D89E-HA[a]	78.9 ± 4.7	29.8 ± 11.1
ToxR-D89A-HA	1.2 ± 0.23	1.7 ± 0.1
ToxR-K92E-HA[a]	36.9 ± 5.2	2.8 ± 0.1
ToxR-K92A-HA	73.8 ± 15.6	33.4 ± 3.9
ToxR-S93P-HA[a]	33.9 ± 4.6	8.1 ± 0.6
ToxR-S93A-HA	104.0 ± 6.9	21.4 ± 3.4
ToxR-K98E-HA[b]	37.3 ± 7.3	17.0 ± 0.5
ToxR-K98A-HA	74.1 ± 6.6	18.0 ± 1.0
ToxR-P101L-HA[a]	34.0 + 11.8	11.2 + 0.8
ToxR-P101A-HA	52.2 ± 16.0	10.3 ± 0.4
ToxR-R103G-HA[a]	24.0 + 4.5	3.9 + 0.1
ToxR-R103A-HA	49.3 ± 17.5	30.0 ± 4.1

a. Data same as that shown in Fig. 1.
b. Data same as that shown in Table S1. Data are from at least two experiments performed in triplicate.
Miller units measure in strains EK410 (O395 Δ*toxR ompU-lacZ*) and EK1072 (O395 Δ*toxR toxT-lacZ*).

epitope-tagged version of TcpP [encoded on pEK41 (Krukonis *et al.*, 2000)]. The interaction between ToxR and TcpP-HSV was assessed using *V. cholerae* membranes harvested after a 6 h IPTG induction step to allow for TcpP-HSV expression and membrane insertion.

We tested the ability of seven ToxR mutant proteins with preferential defects in *toxT* activation to interact with wild-type TcpP: ToxR-D73A, ToxR-D89E, ToxR-K92E, ToxR-S93P, ToxR-K98E ToxR-P101L and ToxR-R103G (six of which lie adjacent to or in the putative wing domain). As a negative control we assessed the levels of interaction between wild-type ToxR and a TcpP mutant protein, TcpP-K101E, shown previously to be defective for ToxR interaction (Krukonis and DiRita, 2003).

Of the ToxR mutants tested, only ToxR-P101L (located at the tip of the wing domain) showed reduced levels of interaction with TcpP, with about 15% of wild-type levels of interaction (Fig. 5). The level of ToxR-P101L interaction with TcpP was as low as the previously characterized weak interaction between ToxR and TcpP-K101E, although ToxR-P101L was somewhat less stable than wild-type ToxR and this may contribute to the reduced level of

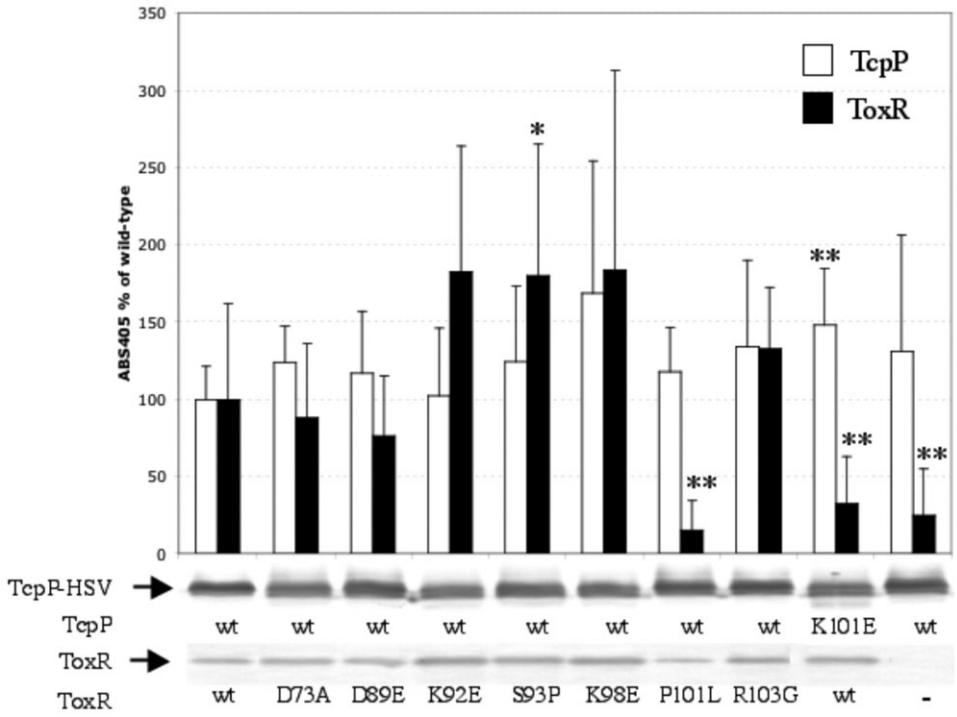

Fig. 5. Assessment of TcpP protein interaction by various ToxR mutant proteins that preferentially affect *toxT* transcription. *V. cholerae* membranes from cells expressing each ToxR mutant derivative were assessed for their ability to interact with wild-type TcpP-HSV or TcpP-K101E. ToxR mutant proteins were expressed from the chromosomal *toxR* locus while TcpP-HSV was expressed from the IPTG-inducible plasmid pEK41 in a Δ*tcpP V. cholerae* background. TcpP-HSV was captured to 96-well microtiter plates using an anti-HSV monoclonal antibody. TcpP capture was assessed by probing the wells with an anti-TcpP polyclonal antibody (Krukonis and DiRita, 2003; Matson and DiRita, 2005), while ToxR co-capture was assessed by probing parallel wells with an anti-ToxR polyclonal antibody. Levels of each ToxR mutant protein and TcpP-HSV were assessed by Western blot on the same protein extracts used for cross-linking. Statistical comparisons were made using the student's *t*-test and compare samples relative to a strain expressing wild-type ToxR and wild-type TcpP-HSV. $*P = 0.05$, $**P < 0.02$.

ToxR/TcpP interaction (Fig. 5). One other mutant, ToxR-D89E, showed a trend towards decreased TcpP interaction, but the defect did not reach statistical significance (Fig. 5, $P = 0.07$). All other ToxR mutants maintained wild-type (or in some cases increased) levels of TcpP interaction (Fig. 5), despite their reduced levels of *toxT-lacZ* activation (Fig. 1, Tables 2 and S1).

As the ToxR/TcpP capture assay is dependent upon lysine-mediated cross-linking, we established a second assay for ToxR/TcpP interaction, a membrane-bound ToxR and TcpP bacterial two-hybrid reporter system. This system is based on the reconstitution of a split adenylate cyclase enzyme of *Bordetella pertussis*, which upon ToxR/TcpP interaction brings the two Cya fragments together leading to cAMP generation and increased β-galactosidase production in *Escherichia coli* (Karimova *et al.*, 1998; 2005). Unlike the cross-linking assay described above, this assay does not rely upon primary amines for DSP-mediated cross-linking. Using the bacterial two-hybrid system we were unable to detect a significant defect in ToxR-P101L interaction with TcpP (Fig. 6), suggesting the defect in interaction in the cross-linking assay may be due to alterations in the orientation of lysines in the vicinity of P101.

These data indicate that ToxR-P101L maintains interaction with TcpP, but in an altered form as compared with wild-type ToxR. It should be noted that the bacterial two-hybrid assay is performed using plasmids expressing ToxR and TcpP in *E. coli* and the levels of ToxR-P101L may be higher than was achieved in *V. cholerae* when ToxR-P101L was expressed from its normal chromosomal locus. None of the other ToxR wing domain mutants tested had a defect in the bacterial two-hybrid assay (Fig. 6).

Two previously characterized TcpP mutants, TcpP-L96S and TcpP-I97T, which failed to interact with ToxR in the capture assay (Krukonis and DiRita, 2003), were completely defective for ToxR/TcpP interaction in the bacterial two-hybrid assay (Fig. 6A), while a third TcpP mutant, TcpP-K101E, completely defective for ToxR cross-linking [Fig. 5 (Krukonis and DiRita, 2003)], was only partially defective in the bacterial two-hybrid assay (Fig. 6A, $P = 0.035$). This mutant may have lost some level of cross-linking in the capture assay (Fig. 5) as a result of substitution of the lysine, which carries a primary amine group. While TcpP-L96S and TcpP-I97T were less stable than wild-type TcpP in this system, it should be noted that some ToxR mutants (ToxR-S93P and ToxR-K98E) were similarly less stable and maintained robust interaction with TcpP (Fig. 6B and D).

Discussion

This manuscript set out to address the differential roles for ToxR at two distinct *V. cholerae* promoters, *ompU* and *toxT*. Using an *ompU-sacB* fusion-based selection strategy, we identified a number of residues in ToxR required for full activation of the *ompU* promoter (Fig. 1). While several of these mutations severely affected *ompU* promoter binding, those in the putative α-loop of ToxR (Fig. 2) had less dramatic DNA-binding defects (Fig. 3A). One particular α-loop mutant, ToxR-V71A, was nearly completely defective for *ompU* activation (3% of wild type) while it maintained intermediate levels of *toxT* activation (15% of wild type, Fig. 1). Thus, this residue in the α-loop may be particularly critical for engagement of the RNAP machinery at the *ompU* promoter. The fact that one other α-loop mutant, ToxR-R65Q, had an *ompU* promoter-binding defect similar to ToxR-V71A (Fig. 3A), yet maintained 35% *ompU* activation also supports the hypothesis that V71 has a specific role in ToxR-mediated *ompU* activation. The importance of residues in the α-loop in *ompU* activation was reiterated with the generation of site-directed α-loop mutations in which one additional α-loop alanine substitution, ToxR-F69A, led to a defect in *ompU* activation (Table 2), but had no effect on *ompU* promoter binding (Fig. 4A). Residue E39 in the neighbouring helix α1 (Fig. 2A and B) also appears to contribute specifically to *ompU* activation (Table S1) and may be part of an RNAP interaction patch for *ompU* activation (Fig. 7).

It should be noted that some mutations in the α-loop affected both *ompU* and *toxT* activation (ToxR-R65A, ToxR-G68A and ToxR-S74A) and two mutants, ToxR-R65Q and ToxR-D73A, affected *toxT* activation preferentially over *ompU* (Fig. 1 and Table 2). Thus the α-loop is not exclusively important for *ompU* activation. However, the ToxR-V71A mutant indicates this residue is particularly important for *ompU* activation as no other α-loop mutant tested had a comparable *toxT* activation defect (97% defective). Other members of the OmpR/PhoB family of winged-HTH activators also have activation-specific mutations identified in their α-loops (Pratt and Silhavy, 1994; Kato *et al.*, 1995). In some cases, suppressor mutations in the gene encoding a component of RNA polymerase can rescue the activation-defective OmpR/PhoB family member (Kato *et al.*, 1996).

Eight ToxR substitution mutants with intermediate defects in *ompU-lacZ* expression (ToxR-R65Q, ToxR-D73A, ToxR-D89E, ToxR-K92E, ToxR-S93P, ToxR-K98E, ToxR-P101L and ToxR-R103G) showed preferential defects in *toxT-lacZ* expression relative to *ompU-lacZ* expression. Six of eight substitutions affect residues adjacent to or within the putative wing domain of the winged-HTH domain of ToxR. The wing domain in this family of proteins has been shown in some instances to be involved in DNA binding, while in other cases it plays a role in protein–protein interactions (Littlefield and Nelson, 1999; Blanco *et al.*, 2002; Krukonis and DiRita, 2003; Yamane *et al.*, 2008). When involved in DNA binding, the

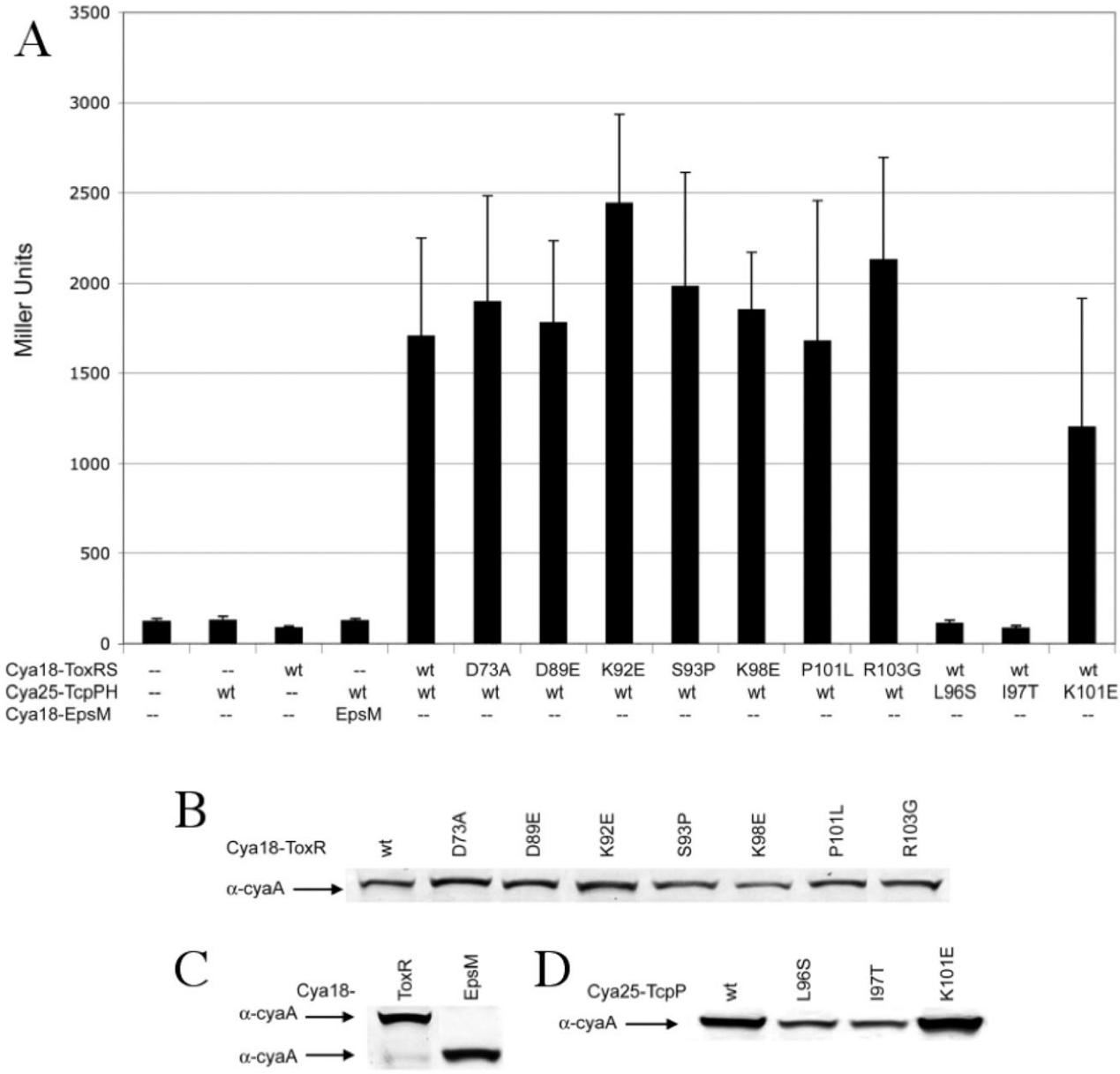

Fig. 6. Assessment of ToxR/TcpP interaction by various ToxR and TcpP mutants using an adenylate cyclase-based membrane-anchored bacterial two-hybrid system.
A. Interaction between wild-type TcpPH fused to the cya25 fragment of *B. pertussis* CyaA and ToxRS fused to the cya18 fragment of CyaA were assessed in strain BTH101. Interaction was determined by measuring β-galactosidase activity produced as a result of reconstitution of a functional adenylate cyclase enzyme. Known non-interacting mutants of TcpP were fused to cya25 as controls. As additional controls cya18-ToxR and cya25-TcpP alone were tested.
B. Western blot analysis of ToxR mutant derivatives fused to cya18.
C. Western blot analysis of wild-type ToxR and EpsM fused to cya18.
D. Western blot analysis of TcpP fused to cya25. All Western blots were probed with rabbit anti-CyaA antibodies (Santa Cruz Biotechnology).

wing binds the minor groove of the DNA helix while the DNA-recognition α-helix (α3, Fig. 2A) recognizes the major groove (Blanco *et al.*, 2002; Yamane *et al.*, 2008). Our findings suggest the wing domain of ToxR is involved in DNA binding (Fig. 3) and may also play a role in TcpP interaction (Fig. 5). We hypothesize that ToxR interaction with TcpP allows ToxR to facilitate activation of the *toxT* promoter. One ToxR wing mutant that affected *toxT* activation preferentially, ToxR-P101L, was defective for this ToxR/TcpP interaction as assessed by a cross-linking assay, with ToxR-P101L expressed from its chromosomal locus (Fig. 5). On the other hand, a plasmid-based ToxR/

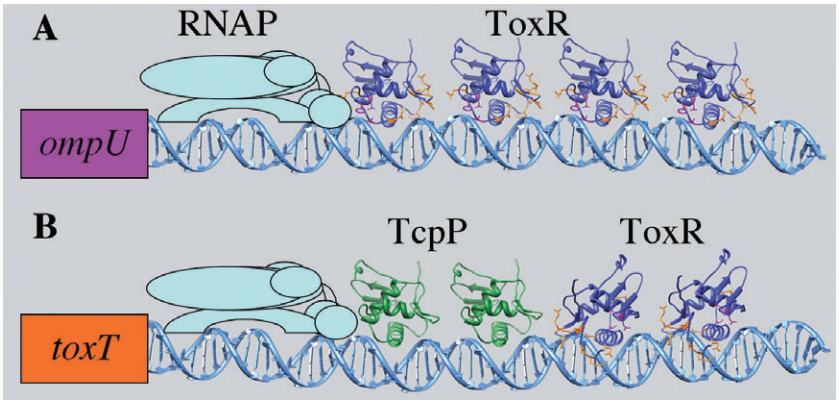

Fig. 7. Model for differential activation of the *ompU* and *toxT* promoters of *V. cholerae* by ToxR.
A. ToxR directly activates the *ompU* promoter in the absence of TcpP using residues F69 and V71 of the α-loop, and possibly E39 of α3, to stimulate RNA polymerase transcription.
B. ToxR activates the *toxT* promoter in conjunction with TcpP by facilitating the ability of TcpP to interact with RNA polymerase. At the *toxT* promoter, ToxR and TcpP may interact via wing–wing contacts between the proteins. Thus, in this model, ToxR is inverted in its binding orientation on the DNA relative to the *ompU* promoter to present the wing face of ToxR to TcpP. Both promoters are shown from a backside view to allow easier visualization of the side-chains that contribute preferentially to *ompU* (magenta) or *toxT* (orange) activation. The DNA template used is from the structure of PhoB bound to DNA (Yamane *et al*., 2008).

TcpP bacterial two-hybrid assay revealed no significant defect for ToxR-P101L/TcpP interaction (Fig. 6). It was notable that wild-type ToxR consistently generated high levels of cAMP in the bacterial two-hybrid assay, resulting in *lacZ* expression levels ranging from 898–2922 Miller Units over 42 measurements, while ToxR-P101L gave more variable *lacZ* expression levels, ranging from 195–2975 Miller Units over 27 measurements (Fig. 6A). Together we take these data to indicate that the ToxR-P101L mutant likely presents a slightly altered conformation of the ToxR wing domain that affects the ToxR/TcpP cross-linking assay more dramatically than the bacterial two-hybrid assay. We hypothesize that in the former assay, this altered wing conformation affects the presentation of a neighbouring lysine residue, which is involved in ToxR/TcpP cross-linking. In support of the hypothesis that the wing of ToxR may interact with TcpP, we have recently uncovered a number of residues in the wing domain of ToxR that affect ToxR/TcpP interactions in the bacterial two-hybrid assay (S.J. Morgan and E.S. Krukonis, unpubl. obs.).

Because no other wing mutants tested had a significant defect in TcpP interaction by either assay, the *toxT*-preferential defects of the other ToxR wing substitutions may reflect their slightly greater defects in *toxT* promoter binding as compared with *ompU* binding (Figs 3 and S1) and/or some other aspect of promoter interaction required for full *toxT* activation. ToxR may have a lower affinity for the *toxT* promoter than the *ompU* promoter, thus the reduced DNA-binding activity of these ToxR wing mutants may largely explain their preferential defects in *toxT* activation. Modest reductions in DNA-binding affinity may also preferentially affect *toxT-lacZ* expression in *V. cholerae* relative to *ompU-lacZ* as ToxR must displace the global regulator H-NS bound to the *toxT* promoter (Nye *et al*., 2000). Alternatively, by affecting the wing domain, these mutants may be altered in their interaction with the minor groove of the DNA in a way that slightly changes the orientation of ToxR on the DNA rather than leading to a major defect in DNA-binding affinity. This could adversely affect the ability of ToxR to efficiently function with TcpP to facilitate *toxT* expression. It remains less clear why the ToxR α-loop mutant derivatives, ToxR-R65Q and ToxR-D73A, showed *toxT*-specific activation defects (Fig. 1 and Table 2), but their *toxT* activation defects were < twofold of their *ompU* activation defects, making them the least dramatic of the *toxT*-specific defects. The ToxR-D73 side-chain is predicted to be oriented towards the wing domain when modelled on other winged-HTH transcription factors (Fig. 2C), although assignment of side-chain orientation in loop regions is tentative. If oriented towards the wing domain, D73 may function along with wing residues of ToxR to enhance TcpP-mediated *toxT* activation. R65 maintains some *toxT*-specific defects even when changed to alanine or leucine (Table 2 and Table S1), although the preference diminishes with these other side-chain substitutions. When bound to the *toxT* promoter with α3 in the major groove it is possible that R65 can contribute to functions specific for TcpP-dependent activation as this side-chain is predicted to extend to the edge of the α-loop (Fig. 2C) and potentially participate in interactions primarily involving the face of ToxR containing the wing domain. Our modelling of ToxR bound to DNA and results with other PhoB/OmpR family members (Blanco *et al*., 2002; Yamane *et al*., 2008) indicate that the α-loop sits 'side saddle' on the DNA (Fig. 7) and interacts with RNAP from

that position. Thus, residues that extend in one direction from the α-loop like F69 and V71 (Fig. 2B) may interact with RNAP while those that extend in the other direction, like R65 and D73 may interact with other components of the activation complex.

In all, these studies define residue V71 of the α-loop of ToxR as being critical for activation of the *ompU* promoter and F69 playing a supporting role in *ompU* activation. Residues adjacent to and within the wing domain of ToxR also affect *ompU* activation, but in a number of cases more strongly affect *toxT* activation. Why some of these ToxR mutations affect *toxT* more dramatically than *ompU* remains to be determined. In at least one case, ToxR-P101L, presentation of the wing domain for ToxR/TcpP interaction was affected (Fig. 5). For the remainder of the ToxR derivatives preferentially defective for *toxT* activation, there was no statistically significant defect in TcpP interaction. Thus, the wing domain of ToxR appears to play a role on both *ompU* and *toxT* activation, but perhaps a dual role in *toxT* activation, DNA binding and TcpP interaction. It is notable that the mutation affecting ToxR/TcpP cross-linking was ToxR-P101L, which is predicted to perturb the structure of the wing domain by eliminating the proline turn residue at the tip of the wing. Mutations in the wing domain of TcpP (also an OmpR/PhoB family member) have been shown previously to affect ToxR interaction (Krukonis and DiRita, 2003). Thus, we present a model where the wing domains of ToxR and TcpP are oriented towards each other on the *toxT* promoter (Fig. 7). ToxR binds the *toxT* promoter near an inverted repeat element (Higgins and DiRita, 1994; Krukonis *et al*., 2000). However, we have evidence that ToxR may bind to an imperfect direct repeat that extends into the 5′ half of this inverted repeat element, rather than binding the inverted repeat element itself (T. Goss and E.S. Krukonis, unpubl. obs.). Thus, we model ToxR on the *toxT* promoter as a head-to-tail dimer with its wing domain oriented towards TcpP (Fig. 7B). At the *ompU* promoter, we orient the promoter proximal ToxR molecule with residues F69 and V71 of the α-loop oriented towards the promoter for interaction with RNA polymerase (Fig. 7A). Orientation of the remaining ToxR molecules on the *ompU* promoter is speculative and future experiments will test the model we propose for ToxR binding throughout the *ompU* promoter (Crawford *et al*., 1998; Li *et al*., 2000).

Experimental procedures

Bacterial strains and plasmids

A list of bacterial strains and plasmids is provided in Table 1. *V. cholerae* were grown in modified LB (with 5 g l^{-1} NaCl rather than 10 g l^{-1}) with 100 μg ml^{-1} streptomycin and 100 μg ml^{-1} ampicillin or 25 μg ml^{-1} chloramphenicol as appropriate.

Construction of an HA-epitope tagged toxR allele

toxR from *V. cholerae* strain O395 was PCR amplified from chromosomal DNA using a primer with a BamHI restriction site and including the *toxR* ribosomal binding site (primer 5′ ToxR-HA) and a 3′ XhoI restriction site (primer 3′ ToxR-HA) to allow in-frame ligation into the HA-tagging vector pcDNA3-HA (Inohara *et al*., 1998). The resulting C-terminally HA-tagged *toxR* allele was confirmed to encode wild-type *toxR* by sequencing. The *toxR-HA* allele was then liberated from pcDNA3-HA by BamHI and ApaI digestion and ligated into the expression vector pSK Bluescript (Invitrogen) for expression in *V. cholerae*. Primers for these studies are listed in Table S2.

Isolation of sucrose-resistant toxR alleles using an ompU-sacB selection

The *toxR-HA* allele was subjected to random Taq polymerase-mediated mutagenesis by standard PCR amplification for 20 rounds of amplification using primers 5′ ToxR-HA and 3′ HA-tag ApaI (Table S2). The *toxR-HA* mutant pool was cut with BamHI and ApaI and ligated into fresh pSK Bluescript digested with BamHI and ApaI. Three pools of ~ 300–400 clones were generated and used to transform the *V. cholerae ΔtoxR ompU-sacB* strain, EK406. Transformants were plated onto LB plates containing 5% sucrose, 100 μg ml^{-1} streptomycin and 100 μg ml^{-1} ampicillin (to select for pSK Bluescript) and 100 μM IPTG to induce *toxR-HA* expression. Within the three pools, the percentage of mutant pSK-*toxR-HA* alleles that resulted in sucrose resistance in strain EK406 was, 2.5%, 4.5% and 6.25% respectively. About 160 colonies from the three pools were picked and screened individually for ToxR-HA expression by Western blot analysis after growth of the strain for 3 h in 3 ml LB + 100 μM IPTG (data not shown). Those that expressed significant amount of full-length ToxR-HA as assessed by anti-HA antibody, were studied further. This amounted to about 25–35% of the total sucrose-resistant clones analysed. Those mutants defective for *ompU-sacB* activation, that expressed full-length ToxR-HA, were sent for sequencing at the University of Michigan Sequencing Core.

Measuring transcriptional activation activity of ToxR-HA derivatives

pSK-*toxR-HA* derivatives encoding various *toxR-HA* alleles were transformed into the *V. cholerae ΔtoxR ompU-lacZ* reporter strain EK410 and the *ΔtoxR toxT-lacZ* reporter strain EK1072. The latter is a *ΔtoxR* derivative of a previously constructed O395 *toxT-lacZ* strain, a kind gift from Dr Claudia Häse (Häse and Mekalanos, 1998 #993). Reporter strains harbouring the various ToxR-HA derivatives were grown in triplicate overnight at 30°C and then diluted 1:50 and grown for 3–4 h at 30°C in the presence of 100 μg $μl^{-1}$ streptomycin, 100 μg ml^{-1} ampicillin and 100 μM IPTG. Cells were harvested and 20 μl or 100 μl were used in a standard β-galactosidase assay {Miller, 1972 #35}. Measurements of *ompU* and *toxT* activation for each ToxR-HA mutant derivative were assessed in at least two separate experiments.

DNA gel mobility shift assays

Promoter-binding assays were performed essentially as described previously (Krukonis *et al*. 2000 #1392). The membranes used were from strain EK459 (*ΔtoxRΔtcpP*) carrying each pSK-*toxR-HA* allele and induced for 4–5 h at 30°C in 500 ml LB pH = 6.5 with 100 μM IPTG. Increasing concentrations of membrane were mixed with either an *ompU* promoter probe extending from −211 to +22 relative to the transcriptional start site, a *toxT* promoter probe extending from −172 to +45 or a negative control *toxT* promoter probe extending from −46 to +45 [lacking the ToxR-binding site (Higgins and DiRita, 1994)]. Probes were labelled by Klenow fill-in of BamHI or SalI digested plasmids with α-^{32}P-dCTP and 3000 cpm of labelled probe were used in each reaction. Relative levels of each ToxR mutant protein were assessed using anti-HA monoclonal antibody (Covance), anti-HA polyclonal antibody (Covance) or anti-ToxR polyclonal antibody. Samples normalized to the same relative concentrations used in the gel-shift assay were boiled in SDS sample buffer and run in a 12% SDS-PAGE gel.

ToxR/TcpP-HSV capture assay

Vibrio cholerae RY1 with various *toxR* alleles recombined at the normal *toxR* locus were transformed with plasmids expressing wild-type TcpP-HSV or the ToxR-interacting mutant TcpP-K101E. Strains were diluted 1:50 from an overnight culture at 30°C and grown for 4–6 h at 30°C in 500 ml LB containing 100 μg ml^{-1} streptomycin, 25 μg ml^{-1} chloramphenicol and 100 μM IPTG. Membranes harbouring ToxR and TcpP-HSV proteins of interest were prepared (Miller *et al*., 1987 #3) and dialysed into HEPES-buffered saline (HBS, 20 mM HEPES pH = 7.0, 150 mM NaCl). Three to five mg ml^{-1} membrane proteins were cross-linked using a 15-fold molar excess of DSP (Pierce) for 30′ at room temp, blocked with 50 mM Tris pH = 7.4 and then solubilized in 1% Triton X-100 (Bio-Rad). The molarity of dialysed membrane preparations was estimated by measuring the protein concentration and assuming a 50 kD average protein size in the total membrane extract. After sonication on ice (3 × 5 seconds), 50 μl membrane extracts was added to microtiter plates coated with mouse anti-HSV antibody (Novagen, coated at 1:500 dilution in PBS) and binding proceeded overnight at 4°C. After washing five times with PBS, wells were incubated with 50 μl a 1:100 dilution of rabbit anti-TcpP antibody or a 1:1000 dilution rabbit anti-ToxR antibody. Primary antibody incubation proceeded from 3 h overnight at 4°C. After five washes with PBS, wells were then incubated with a 1:3000 dilution of goat anti-rabbit-AP conjugated secondary antibody (Zymed) and binding was revealed by addition of 100 μl of the colorimetric substrate PNPP (Sigma) at 4 mg ml^{-1} following sequential washing with PBS (four times) Tris-buffered saline (100 mM Tris pH = 8.0, 150 mM NaCl, one wash). Plates were read at ABS_{405}. Relative levels of each ToxR mutant protein were assessed using an anti-ToxR polyclonal antibody at a 1:1000 dilution. Relative levels of TcpP-HSV in each strain were assessed using an anti-HSV monoclonal antibody (Novagen) at a 1:5000 dilution. The amount of TcpP-HSV or ToxR captured is presented as % of wild type after subtracting out the background signal obtained for each protein in the absence of TcpP-HSV (using the pMMB207 empty vector). Samples diluted on the same day to the same relative concentrations used in the capture assay were boiled in SDS sample buffer and run in a 12% SDS-PAGE gel for Western blot analysis of protein levels in the extracts. Statistical analysis was performed using the student's *t*-test comparing six or nine measurements relative to the strain expressing wild-type ToxR and wild-type TcpP-HSV.

ToxR/TcpP bacterial two-hybrid system

ToxRS and EpsM were PCR amplified from chromosomal DNA and cloned into the pUT18c vector (Karimova *et al*., 2001) 3′ of the cya18 fragment in DHM1. TcpPH was PCR amplified from chromosomal DNA and cloned into the pKT25 vector (Karimova *et al*., 2001) 3′ of the cya25 fragment in DHM1. Point mutations were created in ToxR and TcpP using site-directed mutagenesis (primers are listed in Table S2). Plasmids were transformed into the reporter strain BTH101 and grown at 37°C in the presence of 100 μg ml^{-1} ampicillin and 30 μg ml^{-1} kanamycin. Cultures were induced 16 h at 30°C in LB broth in the presence of 100 μg ml^{-1} ampicillin, 30 μg ml^{-1} kanamycin 0.5 mM and IPTG. 20 μl of culture was used in a standard β-galactosidase assay (Miller, 1972) in a minimum of three separate experiments with three replicates each. Stability of ToxR and TcpP mutants was determined by transforming the cya18-ToxRS mutant plasmid with pKT25 or cya25-TcpP mutant plasmid with pUT18c into BTH101. Cultures were induced 16 h at 30°C in LB broth in the presence of 100 μg ml^{-1} ampicillin, 30 μg ml^{-1} kanamycin, 0.5 mM IPTG and 1 mM cAMP. Samples normalized to the same relative concentrations by OD_{600} were boiled in SDS-sample buffer and run in a 10% SDS-PAGE gel. Samples were probed with anti-CyaA rabbit polyclonal antibody (Santa Cruz Biotechnology).

ToxR mutant modelling

Using a newly developed program I-TASSER (Zhang, 2008; 2009; Roy *et al*., 2010), we threaded the ToxR sequence onto winged-HTH family members with solved structures. I-TASSER made a secondary structure sequence prediction for ToxR and threaded that onto to several best matches in the PDB, compiling the best matching fragments from each structure. In creating the ToxR model, I-TASSER used parts of YycF (from *Bacillus subtilis*), OmpR (*E. coli*), MtrA (*Mycobacterium tuberculosis*), RegX3 (*M. tuberculosis*), ArsR (*Helicobacter pylori*) and VicRc (*Enterococcus faecalis*) as the top 10 threading templates (results at http://zhanglab.ccmb.med.umich.edu/I-TASSER/output/S57294/). It then compiled these best fit pieces and performed energy/hydrogen bonding optimization. The best fit of the ToxR model is to YycF, with 25% sequence identity (with PhoB of *E. coli* as the third best fit). Using the protein structure analysis program Chimera (Pettersen *et al*., 2004), several key residues of ToxR were highlighted to show the predicted orientation of their side-chains to gain insights into their potential effects on ToxR function.

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