Table S1: Activation phenotypes of ToxR derivatives from various mutagenesis studies

ToxR derivative	location	*ompU-lacZ*	*toxT-lacZ*
Controls			
ToxR-HA wild-type	-	100% ± 5.6	100% ± 6.9
pSK	-	0.3% ± 0.03	1.6% ± 0.1
Ottemann mutants			
ToxR-E39K-HA	α1	18.6% ± 1.2	104.5% ± 8.2
ToxR-S55A-HA	β5	94.6% ± 2.4	111.8% ± 3.3
ToxR-R56K-HA	α2	1.2% ± 0.2	2.0% ± 0.1
ToxR-R56L-HA	α2	0.3% ± 0.009	1.8% ± 0.1
ToxR-R65L-HA	α-loop	39.4% ± 0.9	32.3% ± 1.9
ToxR-R84K-HA	α3/DNA-binding domain	0.3% ± 0.03	1.9% ± 0.1
ToxR-R84L-HA	α3/DNA-binding domain	0.3% ± 0.03	1.8% ± 0.04
ToxS-blind mutants			
ToxR-K85E-HA	α3/DNA- binding domain	0.3% ± 0.02	2.0% ± 0.05
ToxR-D89N-HA	between α3 & wing domain	1.0% ± 0.02	2.4% ± 0.1
ToxR-T99M-HA	wing domain	0.4% ± 0.04	2.1% ± 0.1
***toxT-lacZ* white mutants**			
ToxR-R84C-HA	α3/DNA- binding domain	0.3% ± 0.02	2.0% ± 0.05
ToxR-D89Y-HA	between α3 & wing domain	0.5% ± 0.09	2.1% ± 0.2
ToxR-K98E-HA	wing domain	65.7% ± 6.8	25.8% ± 1.2

Table S2: Primers used in this study

primer name	sequence 5' to 3'	purpose
5' ToxR-HA	gggggatcctcaaaagagatatcgatgag	cloning *toxR* into pcDNA-HA and pSK Bluescript
3' ToxR-HA	gggggggctcgagctcacacactttgatggc	cloning *toxR* into pcDNA-HA
3' HA-tag ApaI	cctgacgggcccactagcgaattcatctagaagcg	cloning *toxR* into pSK Bluescript
toxR K85R TOP	catttcgactctgcgcagaatgctcaaagattcg	separation of double mutants
toxR K85R BOTTOM	cgaatctttgagcattctgcgcagagtcgaaatg	separation of double mutants
toxR D89E TOP	caaaatgctcaaagagtcgacaaagtccccac	separation of double mutants
toxR D89E BOTTOM	gtggggactttgtcgactctttgagcattttg	separation of double mutants
toxR R65A	catgactttgtttgggctgagcaaggttttgaagtc	α-loop mutant construction
toxR E66A	gactttgtttggcgagcccaaggttttgaagtcgat	α-loop mutant construction
toxR Q67A	ctttgtttggcgagaggctggttttgaagtcgatgat	α-loop mutant construction
toxR D72A	caaggttttgaagtcgctgattccagcttaacc	α-loop mutant construction
toxR D73A	ggttttgaagtcgatgcttccagcttaacccaag	α-loop mutant construction
toxR S74A	gttttgaagtcgatgatgccagcttaacccaagc	α-loop mutant construction
toxR S75A	gaagtcgatgattccgccttaacccaagccatttc	α-loop mutant construction
toxR D72A BOTTOM	ggttaagctggaatcagcgacttcaaaaccttg	construction of chromosomal mutants
toxR D73A BOTTOM	cttgggttaagctggaagcatcgacttcaaaacc	construction of chromosomal mutants
toxR K92E TOP	cgcaaaatgctcaaagattcgacagagtccccacaatacgtcaaaacggt	construction of chromosomal mutants
toxR K92E BOTTOM	accgttttgacgtattgtggggactctgtcgaatctttgagcattttgcg	construction of chromosomal mutants
toxR S93P TOP	caaagattcgacaaagcccccacaatacgtc	construction of chromosomal mutants
toxR S93P BOTTOM	gacgtattgtgggggctttgtcgaatctttg	construction of chromosomal mutants
toxR K98E TOP	cccacaatacgtcgaaacggttccgaagcgc	construction of chromosomal mutants
toxR K98E BOTTOM	gcgcttcggaaccgtttcgacgtattgtggg	construction of chromosomal mutants
toxR P101L TOP	cgtcaaaacggttctgaagcgcggttacc	construction of chromosomal mutants
toxR P101L BOTTOM	ggtaaccgcgcttcagaaccgttttgacg	construction of chromosomal mutants
toxR R103G TOP	caaaacggttccgaagggcggttaccaattgatc	construction of chromosomal mutants
toxR R103G BOTTOM	gatcaattggtaaccgcccttcggaaccgttttg	construction of chromosomal mutants
ToxR XbaI Fwd	ggggtctagagatgagtcatattggtactaaattcattc	construction of BACTH system
ToxS BamH1 Rev	gggggggatccttaagaattactgaacagtacggtag	construction of BACTH system
TcpP XbaI Fwd	ggggtctagagatggggtatgtccgcgtga	construction of BACTH system
TcpH BamH1 Rev	gggggggatccctaaaaatcgctttgacaggaa	construction of BACTH system
EpsM XbaI Fwd	ggggtctagagatgatgaaagaattattggctcc	construction of BACTH system
EpsM EcoRI Rev	gggggaattctcagcctccacgcttcag	construction of BACTH system
tcpP L96S top	gatgaacataagacgtcgatcgaaaatgtaaag	construction of BACTH system
tcpP L96S bottom	ctttacattttcgatcgacgtcttatgttcatc	construction of BACTH system
tcpP I97T top	gaacataagacgttgaccgaaaatgtaaagttac	construction of BACTH system
tcpP I97T bottom	gtaactttacattttcggtcaacgtcttatgttc	construction of BACTH system
tcpP K101E top	gttgatcgaaaatgtagagttacaaggttatc	construction of BACTH system
tcpP K101E bottom	gataaccttgtaactctacattttcgatcaac	construction of BACTH system

Morgan et al: Supplemental Materials

Additional Results

Characterization of previously identified ToxR mutant derivatives

Over the years a number of ToxR mutant derivatives have been constructed based on homology modeling of specific residues with the OmpR/PhoB of transcription activators (Ottemann *et al*., 1992) or isolated from a mutagenesis screen that affected the accessibility of ToxR to periplasmic proteases (DiRita & Mekalanos, 1991). Comparing activities of the various mutants has been somewhat complicated by the fact that authors focus on different promoters for assessing ToxR activity such as *ctx-lacZ* fusions (DiRita & Mekalanos, 1991, Ottemann et al., 1992, Dziejman *et al*., 1999, Ottemann & Mekalanos, 1995, Kolmar *et al*., 1995), a promoter that is now thought to be directly activated by ToxT rather than ToxR in *V. cholerae* (Champion *et al*., 1997, Yu & DiRita, 2002, Withey & DiRita, 2006). In other cases, *ompU* promoter activity was based on the levels of OmpU or OmpT protein as measured in a semi-quantitative fashion on Coomassie gels. Thus, to standardize comparison of the activities of these various ToxR derivatives, we constructed several of these mutants in our ToxR-HA expression system and tested them for activation of our *ompU-lacZ* and *toxT-lacZ* reporters. In this way we could directly compare activation levels with β-galactosidase assays and determine protein levels by anti-HA Western blots.

Results for various constructed ToxR-HA mutants are presented in Table S1. The initial set of ToxR-HA mutants tested were based on studies by Ottemann *et. al.* (Ottemann et al., 1992) where several ToxR residues conserved in OmpR were targeted for mutagenesis. These mutants had been tested previously for their ability to activate a *ctx-lacZ* fusion in *E. coli*, to alter outer membrane protein profiles from OmpU to OmpT in *V. cholerae* and for the ability to direct cholera toxin production in *V. cholerae* (presumably via the TcpP-dependent activator, ToxT). One should note that since the studies by Ottemann *et. al*., Pfau and Taylor mapped the N-terminus of the ToxR protein and it begins at the second ATG predicted start codon (Pfau & Taylor, 1998), thus we have renumbered the reconstructed ToxR-HA mutants to represent the position of the mutation.

Ottemann *et. al.* noted previously that ToxR-E39K (E51K, by their numbering system) failed to activate a *ctx-lacZ* fusion in *E. coli* and showed an altered OmpU/OmpT profile suggesting ToxR-E39K was defective for transcription activation of *ompU*, but maintained *ompT* repression indicating DNA-binding was unaffected. Our studies confirm that ToxR-E39K has only 19% activity at the *ompU* promoter, yet it still maintains wild-type levels (105%) activity at the *toxT* promoter. Our findings suggest this mutation affects the direct activation of *ompU*, while having no effect on TcpP-dependent activation of *toxT*. This further emphasizes the different mechanism used by ToxR for activation of these two promoters. DNA-binding studies with the ToxR-E39K mutant suggest it is partially defective for DNA binding (Fig. S1). Homology modeling of the ToxR-E39K mutant places it in the first α helix (α1) of the PhoB/OmpR-type DNA-binding and transcription activation domain (Fig. 2). This region does not directly bind DNA, but is a scaffolding helix that is packed against helix 3 (the DNA-recognition helix) and the more C-terminal wing domain (Martínez-Hackert & Stock, 1997, Okamura *et al.*, 2000, Blanco *et al.*, 2002). As such, the ToxR-E39K mutation may affect proper orientation of these two domains as well as the orientation of the transactivating α-loop. Alternatively, portions of the E39 side-chain may directly contact RNA polymerase when ToxR is bound to the *ompU* promoter.

Four *toxR* mutants that Ottemann *et. al.* showed to have little or no activity on the *ompU* or *ctx* promoter and led to almost no cholera toxin (CT) production (ToxR-R56K, ToxR-R56L, ToxR-R84K and ToxR-R84L; (Ottemann et al., 1992)) showed no activity at either the *ompU* or *toxT* promoters using our *lacZ* reporter strains (Table S1). DNA-binding assays demonstrated three out of four derivatives (ToxR-R56L, ToxR-R84K and ToxR-R84L) lack DNA-binding activity for both promoters even though the proteins were stably expressed in the membrane of *V. cholerae* (Figs. S1A & B). One mutant derivative, ToxR-R56K, had weakly detectable binding to the *ompU* and *toxT* promoters (Fig. S1). However, ToxR-R56K failed to activate either promoter. Thus, the level of DNA-binding observed with the ToxR-R56K mutant is insufficient for promoter activation.

The final mutant that we reconstructed from Ottemann's studies was ToxR-R65L which was shown previously to have a partial defect for *ompU* activation based on outer

membrane protein profiles and was 2 to 3-fold decreased for CT production. We also found that ToxR-R65L was partially defective for both *ompU* and *toxT* activation with activation levels of 39% and 32% of wild-type, respectively (Table S1). This mutation lies within the putative α-loop and behaves similarly to our previously generated alanine mutant at this position (ToxR-R65A, Table 2) and mutant ToxR-R65Q from our random mutagenesis and sucrose selection (Fig. 1). ToxR-R65L (Table S1), ToxR-R65A (Table 2) and ToxR-R65Q (Fig. 3) had modest DNA-binding defects that may explain their partially impaired abilities to activate both the *ompU* and *toxT* promoters (Figs. 4, S1 and 1, respectively).

In addition to the mutants generated by Ottemann *et. al.* we also reconstructed three ToxR mutants that were originally isolated from a screen for ToxR derivatives that were susceptible to periplasmic cleavage (resulting in increased activity of an alkaline phosphatase fusion domain) even in the presence of ToxS, that normally protects ToxR from degradation in *E. coli* (DiRita & Mekalanos, 1991). Such "ToxS-blind" alleles were mapped to the cytoplasmic domain of ToxR (DiRita & Mekalanos, 1991). All three ToxS-blind derivatives of ToxR tested (ToxR-K85E, ToxR-D89N and ToxR-T99M) were severely defective for *ompU* and *toxT* activation (Table S1). DNA-gel shift assays revealed that while the proteins are stably expressed, one ToxR derivative (ToxR-K85E) is completely defective for DNA binding activity and derivatives ToxR-D89N and ToxR-T99M maintain only weak binding to the *ompU* promoter (Fig. S1A) and negligible binding to the *toxT* promoter (Fig. S1B).

Characterization of three mutants isolated based on decreased activation of a* toxT-lacZ *fusion reporter. The last group of mutants generated in this study was isolated from a randomly mutagenized pool of pSK-*toxR-HA* alleles for their inability to active a *toxT-lacZ* fusion in *V. cholerae*. For these studies we used a blue/white screen to identify random ToxR mutant derivatives that were defective for activation of the *toxT* promoter. Three mutants were identified in an initial screen that affected *toxT* activation, ToxR-R84C, ToxR-D89Y and ToxR-K98E. ToxR-R84C and ToxR-D89Y were both completely defective for both *toxT* and *ompU* activation (Table S1), whereas ToxR-K98E maintained some activation of both promoters, yet showed a preferential defect (26%

activation vs. 66%) for *toxT* activation as opposed to *ompU*. Like other mutants from our *ompU-sacB* selection that showed preferential *toxT* activation defects, ToxR-K98E affects a residue in the putative wing domain of ToxR (Figure 2C).

Mutant derivatives ToxR-R84C and ToxR-D89Y were unable to bind either the *toxT* or *ompU* promoters (Fig. S1), thus explaining their inability to activate these promoters. Residue K98 clearly plays a role in DNA-binding (Fig. S1), as would be expected by its location in the wing domain (Blanco et al., 2002). However, based on its preferential defect in *toxT* activation as opposed to *ompU* activation, it may also play a role in TcpP-mediated activation of the *toxT* promoter.

Discussion

Over the past several years a number of ToxR mutant derivatives have been generated with various affects on transcription and DNA binding. These studies were performed in various bacterial backgrounds including *E. coli*, *Salmonella typhimurium* and *V. cholerae* (Miller *et al.*, 1987, Kolmar et al., 1995, Dziejman & Mekalanos, 1994, Ottemann & Mekalanos, 1995, Ottemann et al., 1992, Dziejman et al., 1999, Pfau & Taylor, 1996, Pfau & Taylor, 1998, Murley *et al.*, 1999, Krukonis *et al.*, 2000, Crawford *et al.*, 2003). The promoter used in many of these studies was the cholera toxin (*ctx*) promoter, a promoter now believed to be directly activated by the AraC-like transcription factor, ToxT (DiRita *et al.*, 1991, Champion et al., 1997, Yu & DiRita, 2002, Withey & DiRita, 2006). We reconstructed a number of these mutants from various studies using our pSK Bluescript expression vector with a C-terminal HA epitope tag to assess their activation using our *ompU-lacZ* and *toxT-lacZ V. cholerae* reporter strains. These two promoters represent those known to be directly activated by ToxR in *V. cholerae*.

In previous work, Ottemann *et al* reported a number of conserved residues in the DNA-binding and transactivation domain of ToxR were required for *ctx-lacZ* activation in *E. coli*, OmpU and CT production in *V. cholerae* and *ctxA* promoter binding (Ottemann et al., 1992). Our findings using *toxT-lacZ* fusions (a promoter activated prior to CT production) and *ompU-lacZ* fusions in *V. cholerae* were largely in agreement with the previous findings. One difference we found was that ToxR-E39K had an intermediate defect on *ompU-lacZ* expression, but had little effect on *toxT-lacZ* expression (Table S1). Ottemann *et al* reported this mutant to be strongly defective for *ctx-lacZ* activation in *E. coli* and CT production in *V. cholerae* (Ottemann et al., 1992). It is unclear why we saw little to no effect on *toxT-lacZ* activation. It is possible that even in *V. cholerae*, there is a role for ToxR in direct *ctxAB* activation, and this mutation specifically affects *ctxAB* activation, while not affecting *toxT* activation. One other difference we found in our studies was that the ToxR-R84L mutation had no DNA-binding activity for either the *ompU* or *toxT* promoters (Fig. S1). Ottemann *et al* reported this mutant (ToxR-R96L by their numbering system) to maintain DNA-binding activity, yet be unable to activate transcription (Ottemann et al., 1992). Their gel shift assays were performed on *ctxA* promoter fragments, whereas ours used *ompU* and *toxT* promoter fragments. It is appears

this mutation affects binding to the three promoters tested differently. Our data are in agreement that ToxR-R84L is unable to activate transcription.

Regarding the previous findings of DiRita *et al* where they identified a number of ToxS-blind alleles of *toxR* that failed to allow ToxR-ToxS interactions in the periplasm, the three mutants we constructed for testing, ToxR-K85E, ToxR-D89N and ToxR-T99M, behaved much like the mutants previously described (DiRita & Mekalanos, 1991). All three mutants were unable to activate both *ompU-lacZ* and *toxT-lacZ* fusions in *V. cholerae* (Table S1). We also showed that all three mutants were defective for *ompU* (Fig. S1A) and *toxT* promoter binding (Fig. S1B). In the case of ToxR-T99M, this is similar to the defect seen with two mutants identified in our original randomly-mutagenized pools affecting the same amino acid residue, ToxR-T99K and ToxR-T99R. The defect of ToxR-D89N is much more severe than the ToxR-D89E mutant identified in our random screen. Thus, the nature of the side-chain substitution has a great effect on activity of ToxR substitutions at this position. Our randomly isolated ToxR-D89E derivative is a very conservative change, thus it is not surprising that it retains more activity than ToxR-D89N. Given that asparagine (N) is also closely related to aspartate (D), this further suggests the negative charge of residue D89 (which would also be present in the D89E substitution) plays an important role in transcription activation. Finally, ToxR-K85E is a substitution within the DNA-binding helix (α3) of ToxR. By changing a positively charged lysine to a negatively charged glutamate, DNA-binding is completely disrupted and the protein no longer activates transcription. A conservative ToxR-K85R mutation at this position has no effect on transcription activation (data not shown). Thus, all three tested "ToxS-blind" mutants disrupted DNA-binding. Why these substitutions affected the ability of ToxR to interact with ToxS in the periplasm remains unclear. It is possible that DNA-binding facilitates ToxR dimerization and dimerization enhances ToxS interaction. Alternatively, ToxS may act as a chaperone for ToxR until it finds a ToxR dimerization partner (Ottemann & Mekalanos, 1996, Pfau & Taylor, 1998). If ToxR is unable to bind DNA, then once it is released from ToxS it may not stably interact with its ToxR binding partner, rendering the periplasmic domain of ToxR susceptible to protease cleavage.

One last ToxR mutant to discuss is ToxR-K98E. This residue is predicted to lie in the first β-strand of the wing domain (Fig. 2C) and has an intermediate defect in DNA binding to the *ompU* and *toxT* promoters (Fig. S1). This mutation was isolated from a screen for ToxR derivatives with reduced *toxT-lacZ* activation capacity. ToxR-K98E mediates 26% of wild-type activation for the *toxT* promoter and 66% activation for *ompU* (Table S1). One possibility for the preferential defect of ToxR-K98E on *toxT* activation could be an impaired ability to interact with TcpP. However, capture assay analysis and bacterial two-hybrid analysis demonstrated that ToxR-K98E had no defect in interaction with TcpP (Figs. 5 and 6). Thus, while this mutation negatively affects DNA binding, it maintains TcpP interaction. Since the crosslinker used in these studies, DSP, reacts with primary amines, this indicates crosslinking relies upon other lysines of ToxR. This could indicate that K98 and the wing domain of ToxR are not directly involved in ToxR/TcpP interactions, or other neighboring lysines (such as K92 or K102) can also facilitate ToxR/TcpP crosslinking.

Material and Methods:

Isolation of ToxR mutants defective for activation of a* toxT-lacZ *fusion A small pool of *toxR* mutants (about 130) expressed from the IPTG-inducible plasmid pMMB66EH (Fürste *et al.*, 1986) were screened for their ability to activate a *toxT-lacZ* reporter in *V. cholerae*, strain EK816 (Table 1). After transforming EK816 with the mutant pool, cells were grown for 1 hr in LB prior to plating on LB Agar supplemented with 10μM IPTG, 100μg/ml ampicillin (to select for the plasmid pMMB66EH-*toxR*) and 40μg/ml X-gal at 30°C overnight. 24 white colonies were picked and analyzed for expression of full length ToxR protein by Western blot analysis using an anti-ToxR antibody. Plasmids expressing full-length protein were subjected to sequencing analysis at the University of Michigan Sequencing Core. Mutants of interest were removed from plasmid pMMB66EH-*toxR* and cloned into pcDNA3-HA and then pSK Bluescript as described for mutants from the sucrose selection strategy.

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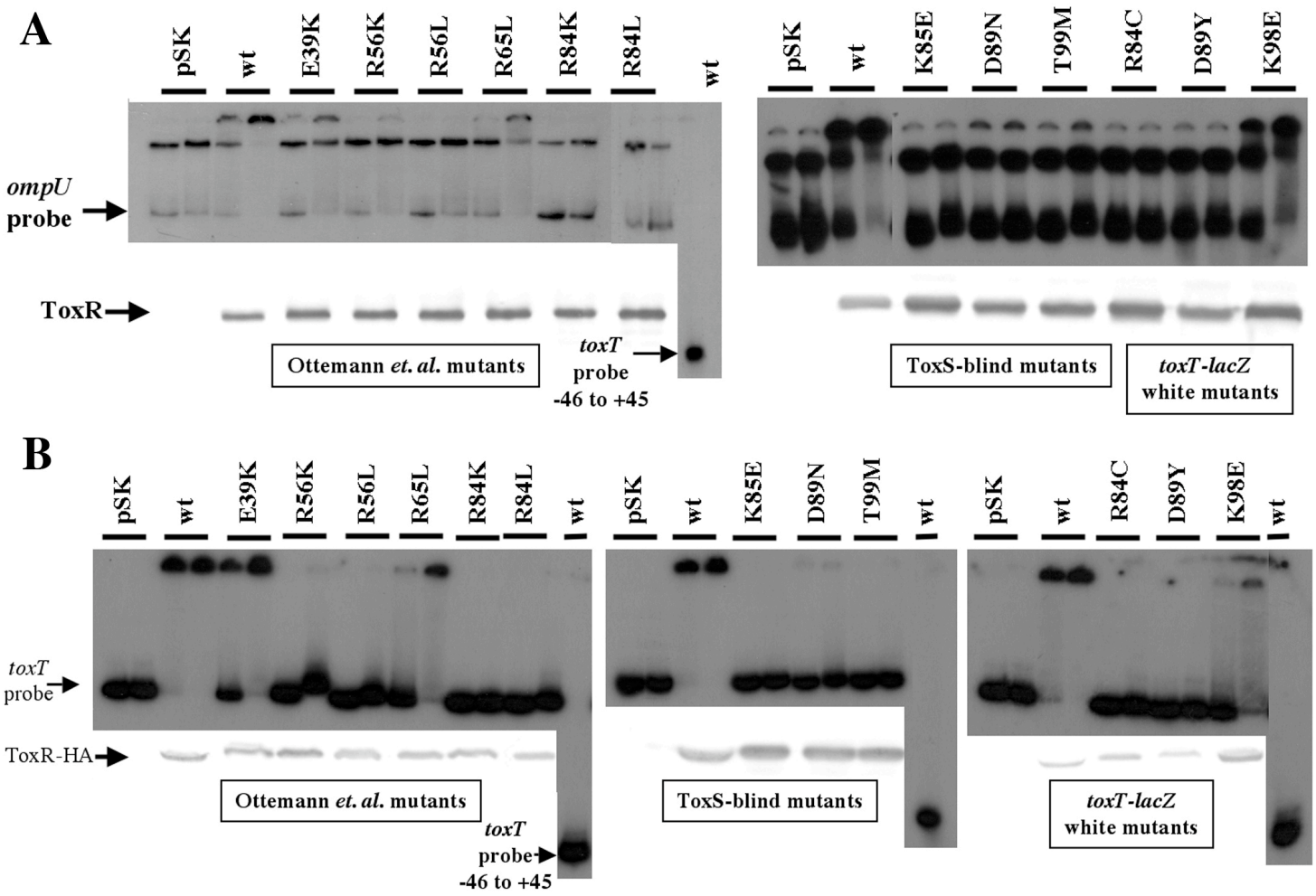

Figure S1: Gel-shift assays to assess promoter recognition by various ToxR mutant proteins identified previously (Ottemann et al., 1992, DiRita & Mekalanos, 1991) or in a random mutagenesis *toxT-lacZ* blue-white screen (this study). *V. cholerae* membranes prepared from a *ΔtoxRΔtcpP V. cholerae* strain (EK459) expressing each mutant ToxR derivative (or wild-type ToxR-HA) were mixed at 0.05 and 0.25 mg/ml with radiolabeled *ompU* promoter DNA (A) or 0.1 and 0.5 mg/ml with *toxT* promoter DNA (B) prior to running samples in a non-denaturing PAGE.