ToxR Proteins with Substitutions in Residues Conserved with OmpR Fail To Activate Transcription from the Cholera Toxin Promoter

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The ToxR protein of Vibrio cholerae is an integral membrane protein that coordinately regulates the expression of virulence genes required for successful infection. ToxR has been shown to bind directly to and activate transcription of the cholera toxin (ctx) promoter. Within the amino-terminal cytoplasmic region of ToxR, several amino acids are strictly conserved among ToxR, OmpR, and the other members of a family of bacterial regulatory proteins. To better understand the function of this region, two approaches were taken: conserved residues were changed by site-directed mutagenesis, and random mutations that eliminated ToxR-mediated transcriptional activation were isolated. Several classes of mutations were identified: those that abolish promoter DNA binding and transcriptional activation (toxR R96K, toxR R65K, and toxR R65L), those that abolish transcriptional activation but retain the ability to bind promoter DNA (toxR R96L), and those that have an intermediate phenotype (toxR R77L, toxR E51K, and toxR E51D). The toxR E51K allele had reduced activity in both Escherichia coli and V. cholerae but also exerted a dominant-negative effect over wild-type ToxR when assayed in V. cholerae. This result provides additional evidence that ToxR acts as an oligomer in the transcriptional activation process. From this mutational analysis of conserved amino acid residues within the OmpR-homologous region of ToxR, we conclude that this region is essential for transcriptional activation at the level of DNA binding and other steps that lead to activation of the ctx promoter.

Vibrio cholerae is a gram-negative bacterium that causes the diarrheal disease cholera. To colonize the human intestine and establish an infection, the organism expresses a group of virulence factors including cholera toxin, the toxin-coregulated pilus, various outer membrane proteins, and an accessory colonization factor (22, 23, 30, 38). These virulence factors are under the coordinate regulation of the ToxR protein, a 32,527-Da integral membrane protein that is required for full virulence of the organism (13, 24). The ToxR protein has been shown to increase expression of cholera toxin by directly increasing transcription from the ctxAB promoter (22). The increase in transcription is mediated by direct ToxR binding to a series of three to eight copies of a TTTTGAT repeat motif found immediately upstream of the −35 region of the ctx promoter (24).

The ToxR protein is located in the inner membrane with approximately two-thirds of its structure located in the cytoplasm and one-third located in the periplasm. The toxR gene is transcribed in an operon with the toxS gene (21). ToxS, which is essential for ToxR function when ToxR is expressed from its own promoter, is also an integral membrane protein that is primarily located in the periplasm (7). Evidence from experiments analyzing the behavior of different ToxR-alkaline phosphatase fusion proteins indicates that the ToxR and ToxS proteins interact directly (7). This interaction probably occurs in the periplasm and involves the portion of ToxR that is most proximal to the cytoplasmic membrane.

The periplasmic domain of ToxR is thought to be involved in sensing the environment. Fusion proteins in which part of the periplasmic portion have been replaced by alkaline phosphatase (ToxR-PhoA) no longer respond to certain environmental signals (24). This could be because the periplasmic domain of ToxR is absent in these fusion proteins or because such fusion proteins adopt an activated conformation that bypasses environmental signalling events. On the basis of the dimerized state of ToxR-PhoA fusion proteins, it has been suggested that this activated conformation may be associated with dimerization of ToxR within the membrane (7, 24).

The cytoplasmic portion of ToxR is homologous to a family of transcriptional activators (Fig. 1) (24). This family, called the two-component family, is involved in prokaryotic signal transduction of a wide variety of environmental signals (reviewed in references 1 and 35). Members of this family consist of two proteins: a sensor and an effector. The sensor responds to environmental conditions and transmits a signal, often by phosphorylation, to modify the activity of the effector. This modified effector protein then carries out the response to the original signal, usually by altering gene expression. The effector proteins show sequence homology in two domains: an amino-terminal domain that is modified by the sensor and a carboxy-terminal domain that is required for transcriptional regulation. The other effector proteins show homology between the cytoplasmic amino terminus of ToxR and the carboxy terminus of a subgroup of these effector proteins, including OmpR, VirG, PhoP, and PhoB (20, 24). This homologous region is thought to be involved in DNA binding and transcriptional activation (24, 36) and is referred to herein as the OmpR-homologous region.

The carboxy-terminal, periplasmic domain of ToxR is presumed to be involved in sensing the intestinal environment and by some subsequent process to activate the

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OmpR-homologous domain of ToxR to mediate increased transcription. Little is known about the nature of this activation process and specifically about the roles of various OmpR-homologous amino acid residues in DNA binding and transcriptional activation. This study was undertaken to further define the structure and function of the OmpR-homologous domain in the ToxR protein. Two approaches were used to study this region: site-specific substitution of amino acid residues that are strictly conserved within this family and screening of mutations that abolished the ability of ToxR to activate cpx transcription. The results presented here indicate that this region of the ToxR protein is involved in both promoter binding and transcriptional activation and that the conserved amino acid residues play a key role in both of these processes.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** The Escherichia coli and V. cholerae strains and plasmids used in this study are listed in Table 1. Strains were grown in LB medium (19) at 30°C. Strains were maintained at -70°C in LB medium containing 25% glycerol. Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; tetracycline, 12 μg/ml; kanamycin, 30 μg/ml; chloramphenicol, 20 μg/ml; and streptomycin, 100 μg/ml.

Transfer of IncP plasmids from E. coli to V. cholerae was accomplished by triparental mating (18) with plasmid pRK2013 (8) for mobilization.

**Recombinant DNA techniques.** Cloning of restriction fragments and DNA sequence analysis were done by standard protocols (32). The DNA sequence was analyzed by using dideoxy kits supplied by Amersham and the manufacturer’s protocols. Enzymes were purchased from New England BioLabs, Bethesda Research Laboratories, Inc., and Boehringer Mannheim.

**Oligonucleotide-directed mutagenesis.** Site-specific mutations were introduced into the toxR gene by using the Amersham oligo-directed in vitro mutagenesis system, version 2 (Amersham), which is based on the method of Eckstein et al. (37). Oligonucleotides used in this study to generate mutations were synthesized on an Applied Biosystems 381A DNA synthesizer and contained the following sequences: mutant E51D, 5’ TCGGCTGTCGGTGTGCCC 3’; mutant E51K, 5’ TCGGCTTTTTGTCGGTGC 3’; mutant S67A, 5’ GCAGATCATTGCCATTTCTT 3’; mutant R96K, 5’ GCAAGTATCTGTCGGTGC 3’. These mutations were generated in plasmid pMJ18ToxR. Double-stranded replicative-form DNA was prepared by standard methods (32), and the 750-bp EcoRI-NcoI fragment of pMJ18ToxR was subcloned into EcoRI-NcoI-digested pVM16 to create a full-length toxR gene containing the desired mutation.

**Isolation of toxR mutants that abolish ToxR activity.** Mutagenesis of toxR::phoA was performed by growing plasmid pToxRPhoA-S (7) in E. coli ES942, which contains the
mutD5 mutation (6). Mutagenized plasmid pools were used to transform strain CC118 (17) to ampicillin and kanamycin resistance. Colonies that were blue on LB plates containing the alkaline phosphatase chromogenic substrate 5-bromo-4-chloro-3-indolyl-phosphate-p-toluidine salt (X-Phos; Bachem) (20 μg/ml) were used as a source of plasmid DNA to transform strain VM2 (22). Fifteen colonies that were white on MacConkey-lactose agar plates (Difco) were picked as potential toxR mutants that could no longer activate transcription from the ctx promoter. These were analyzed for the presence of full-length ToxR-alkaline phosphatase protein by utilizing Western immunoblots with rabbit anti-alkaline phosphatase polyclonal serum. Plasmid DNA was isolated from VM2 strains that produced full-length ToxR-alkaline phosphatase, and DNA sequence analysis was performed to determine the location of the mutation(s).

**Isolation of intragenic suppressors of toxR mutant R96K.** Plasmid pVM16 R96K DNA was isolated from E. coli ES942 after overnight growth and used to transform strain VM2 to ampicillin resistance. Eighteen colonies that were blue on the β-galactosidase chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Boehringer-Mannheim) were selected from a background of 20,000 white colonies. Plasmid DNA from two such colonies was isolated, and DNA sequence analysis was performed to determine the location of the reversing mutation(s).

**Antiserum and antibodies.** Antiserum directed against the ToxR protein was prepared as follows. The ToxR protein was fused with maltose binding protein (MBP) by use of the protein fusion and purification kit supplied by New England BioLabs. Fusion proteins were created by digesting plasmid pVM16 with Clai and then digesting the resulting DNA fragments with S1 nuclease to generate blunt ends. These fragments were then digested with SalI, and a 2,430-bp (Clai)-SalI DNA fragment containing the toxR gene was purified by agarose gel electrophoresis and extraction with the GeneClean DNA affinity kit (Bio 101, Inc.). This fragment was subcloned into plasmid pMal-c (New England BioLabs) digested with Stai and SacI by overnight ligation at 16°C in the presence of T4 DNA ligase and then used to transform E. coli DH5α (12, 31). Transformants were plated on LB agar containing ampicillin and X-Gal, and plasmid (pKO1) DNA was isolated from white colonies. The presence of the 2.4-kb insert that created an MBP-ToxR fusion protein was verified by digesting the plasmid DNA with HinfIII and by analyzing proteins from DH5α strains harboring pKO1 treated with isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma) to induce expression of the fusion protein from the Ptac promoter. Strains that contained the MBP-ToxR fusion protein expressed an 80-kDa protein that was present only in IPTG-induced cultures and was recognized by rabbit anti-MBP serum in Western blots (data not shown). To prepare protein for immunizing injections, an overnight culture of DH5α(pKO1) was diluted 1 to 100, 2 mM IPTG was added, and the culture was grown for 2 h at 30°C. Cells were collected by centrifugation, resuspended in 2 ml of 2 × SDS-sample buffer, and boiled for 5 min (2). Samples of 1 ml were loaded onto two 10% acrylamide gels, and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The 80-kDa induced protein band was identified on Coomassie blue-stained gels by comparison with uninduced cultures, and this band was excised from the gel and washed in three changes of 15 ml of distilled water. This acrylamide band was pulverized, mixed with 0.5 ml of complete Freund’s adjuvant (Sigma), and used to inject two female New Zealand White rabbits (aged 3 months) subcutaneously and intramuscularly. After 5 and 9 weeks, the animals were given booster injections with acrylamide gel slices containing the MBP-ToxR protein prepared as described above, except that Freund’s adjuvant was not added. Animals were bled once per week, starting at 2 weeks after the first booster injection and continuing until 6 weeks after the second booster injection, when both rabbits were exsanguinated. Serum was stored at 4°C in the presence of 0.1% sodium azide until used.

**Western blot analysis of ToxR proteins.** One-milliliter cultures of VM2 harboring pVM16 mutant derivatives were grown overnight and harvested by centrifugation. The cell pellets were resuspended in 0.4 ml of 2× sample buffer and used for Western blotting as previously described (7). Rabbit anti-ToxR serum (see above) was used at a 1:1,000 dilution. The signal seen on Western blots was shown to be approximately proportional to the amount of ToxR protein present by demonstration that a dilution of the protein samples led to a corresponding decrease in this signal.

**Cholera toxin enzyme-linked immunosorbent assays and protein analysis.** V. cholerae strains were grown in LB broth at 30°C for 18 h with aeration as described previously (38). Cells were collected by centrifugation, resuspended in 0.4 ml of 2× sample buffer, and boiled for 5 min. Then, 10 μl of this extract was electrophoresed on SDS–PAGE (11% polyacrylamide), and proteins were visualized by staining with Coomassie brilliant blue as described previously (2). Supernatants of the same cultures were used for GM-1 enzyme-linked immunosorbent assays of cholera toxin (14).

**Preparation of membrane fractions containing ToxR.** E. coli membrane fractions containing wild-type and mutant ToxR proteins were prepared as previously described (24), except that VM2 cells harbored both the plasmid pVM1, which expresses the ToxS protein, and the pVM16 mutant derivative plasmid. Total protein concentration was determined by reading the optical density at 280 nm (OD280) of membrane fractions.

**DNA gel mobility shift assays.** Retardation of the mobility of radioactively labelled DNA fragments was performed as described previously (24) with the following modifications. Radioactively labelled DNA restriction fragments containing the ctx promoter were generated by digesting pJM17 with XbaI and end labeling the resulting fragment with the Klenow fragment of DNA polymerase I and [32P]dCTP. This was then digested with HhaI and DdeI to generate two labelled fragments; one 254 bp fragment that contains the ctx promoter region and ToxR binding site and one 135-bp fragment that contains internal ctxA sequences. This DNA was incubated with membrane fractions containing a final protein concentration of 500 μg/ml at 30°C for 30 min in DNA binding buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% glycerol, 50 μg of bovine serum albumin per ml, 5 mM NaCl, 50 mM KCl), and then electrophoresed in Tris–borate–EDTA–5% acrylamide gels (4% cross-link).

## RESULTS

**Construction of mutations in toxR and function in E. coli.** To analyze the function of the OmpR-homologous region of ToxR, conserved residues were changed by site-directed mutagenesis. Amino acid residues selected for mutagenesis are highly conserved within the OmpR family, and these were substituted with a chemically similar amino acid and a chemically different one. The amino acid substitutions characterized in this study are glutamic acid 51 to aspartic acid or

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*Note: The text is a transcription of the original document, with some formatting adjustments for readability.*
lysine, serine 67 to threonine or alanine, arginine 68 to lysine or leucine, and arginine 96 to lysine or leucine (Fig. 1). All mutations were generated in pM13mp18ToxR and then cloned into pVM16 (Table 1). This plasmid contains the toxR gene under the transcriptional control of the tetracycline resistance promoter. Mutations are designated in the single-letter amino acid code as follows: wild-type amino acid, residue number, new amino acid (e.g., E51K).

ToxR 47

GSNSRILMLLQARPNEVSLRNHDHPWREGQFEVVD...SSLTQAISTLRKLMKDS.TKSFQYVTVPKRGY

CadC 28

LEPRLIDLLVFAQHSGELVSLRDLEDNWKRS...IVTN.HVTQSISELRLKSLNDNEPSVYIAVTVPKRGY

PhoP 162

LTKEBFELLLYLGRKGVRLTDDLSSAWNYD...FAGDTRIVDVHISHLRTPKINNOKPIYIKIKIRGLGY

VirG 156

LTAGEFNLVAFLEKPRGDLSEQ...LAAVREDEYD...RSIDVLFLPRLKKEAD...PSPSLKQKARTAGY

OmpR 161

LTSGEPAVLKKALVSHREPFLRDKin...RGEY...SMAL...RISIDVQISLRLMRVEED...PAHPRYIQTVWGLGY

PhoB 155

MGPTBFKLLHFMTKPKSREQLLNNHVWGTNYV...VED.RTVDVHRRLRKAEPGHIDRM.VQTVRGTGY

Consensus

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![Alignment of toxR and PhoB sequences.](http://jb.asm.org/)

FIG. 1. Amino acid sequence homology between ToxR and bacterial transcriptional regulators. Amino acid residues are aligned to maximize common residues between ToxR and proteins CadC (39), OmpR, PhoB, PhoP (20), and VirG; homologies are taken from Miller et al. (24) and the National Center for Biotechnology Information BLAST network service. Residues common to ToxR are indicated by boldface type, and a consensus sequence for residues common to at least four of six members is indicated. Residues mutated in this study are designated by arrows above the alignment and by arrows plus ToxR residue numbers below the alignment. Mutations in PhoB and OmpR are indicated with asterisks above their respective sequences (see Discussion).

cctx transcription. Changing serine 67 to either a threonine or an alanine had no effect on ToxR activity. In contrast, changing the adjacent arginine residue at position 68 to either lysine or leucine had a drastic effect and abolished ToxR-mediated transcriptional activation. Similarly, substitution of arginine 96 with either lysine or leucine resulted in the elimination of ToxR-mediated transcriptional activation.

toxR mutants retain the ability to produce full-length gene products. To determine whether these toxR mutants were still able to produce full-length ToxR protein, total cell protein was prepared from strain VM2 containing pVM16 mutant derivatives. Proteins were separated by SDS-PAGE and then analyzed by Western blotting with rabbit polyclonal anti-ToxR serum (Fig. 2). The majority of the mutants retained the ability to make stable full-length ToxR protein, with one exception: VM2 cells with plasmids containing the E51K mutation contained significantly less ToxR protein. Western blots were repeated with total cell protein from V. cholerae strains containing the pVM16 mutant derivatives. The mutants that produced full-length protein in E. coli also

| Table 2. Activation of cctx::lacZ fusion in E. coli VM2

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<th>Plasmid</th>
<th>Activity (U)</th>
<th>% of wild-type activity</th>
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<td>pVM16</td>
<td>370</td>
<td>100</td>
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<td>pVM16 E51K</td>
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<tr>
<td>pVM16 E51D</td>
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<tr>
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<td>pVM16 R68K</td>
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<td>4</td>
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<td>pVM16 R77L</td>
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<tr>
<td>pVM16 R96L</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>pVM16 R96K</td>
<td>2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>pVM16 R96K M98K</td>
<td>185</td>
<td>50</td>
</tr>
</tbody>
</table>

| **Reported activities are β-galactosidase activities and are expressed in Miller units (19). The background activity of strain VM2 (30 Miller units) was subtracted from activities given above.**
In V. cholerae, the toxR E51K allele was able to produce increased amounts of full-length gene product in V. cholerae (data not shown).

Isolation of an intragenic suppressor of R96K. To identify intragenic suppressors of one of the toxR mutants, second-site mutations that restored the ability of ToxR to activate transcription were generated. Plasmid pVM16 containing the toxR R96K allele, which has less than 1% of wild-type activity, was mutagenized by growth in E. coli ES942 and used to transform strain VM2. Transformants were plated on media containing X-Gal, and 18 blue colonies that were now able to activate ctx transcription were identified from a background of 20,000 white colonies. Two such revertants were used as a source of plasmid DNA for sequence analysis. Both revertant plasmids retained the first mutation coding for R96K and contained a transversion that resulted in an amino acid change at position 98 from methionine to lysine. This restored ToxR ability to activate transcription from the ctx promoter to 50% of the wild-type level (as compared with less than 1% of wild-type activity for the R96K mutation) (Table 2).

Function of the mutant ToxR proteins in V. cholerae. The ToxR protein controls the expression of several proteins in V. cholerae, including the differential expression of two outer membrane proteins, OmpU and OmpT, and the expression of cholera toxin (22, 23). In addition, ToxR interacts with ToxS in this environment. To assess whether the mutant proteins were still able to function in a homologous system, they were analyzed in V. cholerae. pVM16 derivatives containing the mutant toxR genes described above were mated into V. cholerae JJM43, a derivative of strain 0395 that contains a 25-bp deletion in the amino terminus of toxR and is phenotypically ToxR null. JJM43 derivatives containing plasmids with mutant toxR alleles were then analyzed for the ability to produce cholera toxin B subunit and for the presence of outer membrane proteins. In general, the abilities of the various ToxR mutants to activate transcription in E. coli correlated with their abilities to increase expression of OmpU and cholera toxin (3). On the basis of these observations, the mutations can be divided into three categories: mutants that behave like wild-type ToxR (S67A and S67T), mutants that behave like ToxR null mutations (E51K, R68K, R68L, R96L, and R96K), and mutants that have an intermediate phenotype (E51D and R77L). Both mutant ToxR proteins that have an intermediate phenotype have less activity in E. coli than in V. cholerae. This may be due to an intrinsic increase in stability of the ToxR protein in a V. cholerae background, as was observed with Western blots of the toxR E51K allele.

One ToxR mutant is dominant over the wild type. To assess the function of these mutant toxR alleles in relation to that of wild-type toxR, plasmids containing the mutant alleles were mated into V. cholerae 0395, which contains the wild-type toxR gene on the chromosome. Supernatants of these strains were then analyzed for cholera toxin levels. 0395 containing the pVM16 mutant derivatives produced wild-type levels of cholera toxin (approximately 3,000 ng/OD600 unit) with one exception: 0395 containing the toxR E51K allele produced 10-fold less cholera toxin per OD600 unit (320 ng/OD600 unit).

ToxS increases the ability of ToxR to bind DNA in a gel shift assay. The ToxR protein binds directly to the cholera toxin promoter (24). In previous studies the assay for ToxR-mediated gel mobility shift of DNA was performed with total membranes from cells expressing ToxR-alkaline phosphatase fusion proteins (24). When these assays were repeated with wild-type ToxR instead of the fusion protein, ToxS was needed to facilitate ToxR binding to the ctx promoter at low protein concentrations. When membranes were prepared from E. coli cells containing both ToxR and ToxS, the ctx promoter fragment could be shifted with 125 μg of total protein per ml (Fig. 4). In contrast, when membranes were prepared from cells containing only ToxR, concentrations of greater than 500 μg of total protein per ml were required to shift the ctx promoter DNA.

Ability of mutant ToxR proteins to bind and shift ctx promoter DNA. To distinguish whether the inability of the mutant ToxR proteins to activate transcription resides in a defect in promoter binding or whether these proteins re-
FIG. 4. ToxS increases the ability of ToxR to bind ccr promoter DNA in a gel mobility shift assay. DNA fragments from pJM17 were incubated with VM2 membranes made from cells expressing ToxR or both ToxR and ToxS. The locations of the 254-bp ccr promoter fragment, the 135-bp internal fragment which does not contain the ToxR-binding site, and the shifted fragment are indicated to the left. The lanes contain DNA mixed with membranes from VM2 cells harboring the following in the amounts given within parentheses: 1, DNA only; 2, pVM51 plus pBR322 (1,000 μg/ml); 3, pVM16 (1,000 μg/ml); 4, pVM16 (500 μg/ml); 5, pVM16 (250 μg/ml); 6, pVM16 (125 μg/ml); 7, pVM51 plus pVM16 (1,000 μg/ml); 8, pVM51 plus pVM16 (500 μg/ml); 9, pVM51 plus pVM16 (250 μg/ml); 10, pVM51 plus pVM16 (125 μg/ml).

FIG. 5. Ability of ToxR mutants to bind and shift ccr promoter DNA. The locations of the 254-bp ccr promoter fragment, the 135-bp internal fragment, and the shifted band are indicated to the left. The lanes contain DNA mixed with membranes from VM2 cells harboring pVM51 and the following: 1, DNA only; 2, pBR322; 3, pVM16; 4, pVM16 E51K; 5, pVM16 E51D; 6, pVM16 S67A; 7, pVM16 S67T; 8, pVM16 R68L; 9, pVM16 R68K; 10, pVM16 R77L; 11, pVM16 R96L; 12, pVM16 R96K; 13, pVM16 R96K M98K.

DISCUSSION

The ToxR protein is central for the control of virulence factor expression in V. cholerae, and its position in the membrane allows both sensing of the external environment and regulation of gene expression. In this study we have mutated conserved residues in the cytoplasmic OmpR-homologous region of ToxR to determine the function of this domain in the transcriptional activation process. Transcriptional activation can be thought of as a two-step process: (i) recognition and binding of the promoter and (ii) stimulation of transcription of the gene by interaction with RNA polymerase. Thus, for a transcriptional activator protein, one would expect to find activation-abolishing mutations that fall into both classes; that is, mutations that affect DNA binding and recognition and those that affect only the RNA-polymerase interaction. The majority of the residues altered in this study result in proteins that no longer bind DNA (ToxR E51K, ToxR R68L, ToxR R68K, and ToxR R96K). These mutations could result in either an alteration in the actual promoter recognition domain of ToxR or a more general effect on ToxR structure, such as a gross conformational change or disruption of an active oligomeric state.

The other expected class of mutations would retain the ability to bind DNA but would no longer interact productively with RNA polymerase. Several such mutants, including the positive control mutants of the cyclic AMP binding protein (3,9,16) and the FNR protein (40), have been described. One of the mutants generated in this study, ToxR R96L, has this same phenotype. Based on the characteristics of this mutant, this region of the protein may interact directly with RNA polymerase. Indeed, two mutations in the ToxR homologs OmpR and PhoB, ompR179 and phoB562, correspond to this same region of ToxR (residues 90 through 98 of ToxR; Fig. 1) and alter the transcriptional regulation phenotypes of their proteins (11, 27, 33, 41). Moreover, the ompR179 gene product has been shown to retain the ability to bind DNA, although its specificity is altered (25). The OmpR protein has been shown to interact directly with the alpha subunit of RNA polymerase (34). Thus, this region in ToxR and in the related regulatory proteins is vital for its correct function but may contribute at a step after DNA binding. Curiously, a more conservative substitution within this region of ToxR (the toxR R96K mutation) eliminated DNA binding. Thus, an alternate possibility is that this region directly contacts DNA and this contact is abolished by a lysine at this position. Further mutational analysis to differentiate between these two possibilities is under way.

Changes at glutamic acid 51 also dramatically affected the ToxR protein, but this effect was more pronounced in the

or V. cholerae (E51D, S67A, S67T, and R77L) retained the ability to bind and shift DNA. The pseudorevertant R96K M98K regained the ability to bind and shift DNA. In contrast, mutants that lost the ability to activate transcription (E51K, R68L, R68K, and R96K) lost the ability to bind and shift DNA. One mutant that lost the ability to activate transcription, R96L, did retain the ability to bind to promoter DNA. This mutant protein could not activate transcription in either E. coli or V. cholerae but was able to bind DNA as well as wild-type ToxR in our assay. In addition, the ToxR E51K protein that exhibited a dominant-negative effect over wild-type ToxR was unable to bind DNA. However, this observation is complicated by the fact that only very low levels of this protein accumulate in E. coli membranes.

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heterologous organism *E. coli*. Both amino acid substitutions at this position resulted in inactive proteins when assayed in *E. coli*, but each had a different phenotype when assayed in *V. cholerae*. The toxR ES1K allele produced detectable protein only in *V. cholerae*; although this protein displayed no activity on its own, it exerted a dominant-negative effect over wild-type ToxR. This dominant-negative effect may be exerted through the formation of defective ToxR-ToxR ES1K heterodimers. Evidence that ToxR may act as a dimer has been reported (24).

In addition, this study has further defined the role of ToxS. Previous work had demonstrated that ToxS was necessary for ToxR function only when the concentration of ToxR protein was low and that ToxS interacted directly with ToxR. It has been postulated that ToxS functions to facilitate formation of ToxR homodimers (7). Indeed, ToxS functions to prevent degradation of ToxR and ToxR-alkaline phosphatase fusion proteins (7, 28) and may accomplish this by driving the ToxR protein into its most stable conformation as a dimer or multimer. In this study, we show that ToxS functions to decrease the overall concentration of ToxR membranes required to bind and shift ctc promoter DNA. The ToxS effect on ToxR DNA binding capability may be similar to its effect on stability: ToxS-driven formation of ToxR homodimers establishes a ToxR conformation that is both more stable and better able to bind DNA.

In conclusion, this preliminary mutational study has demonstrated that the OmpR-homologous domain of ToxR is important for its transcriptional regulation function. This domain contains many residues that are highly conserved within this OmpR-homologous family, and changing several of these residues severely impairs ToxR-mediated transcriptional activation. Whether this conserved region contains separate DNA binding and RNA polymerase interaction motifs remains to be seen; however, the results presented in this study support the importance of this region for both functions.

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