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Activation of both *acfA* and *acfD* transcription by *Vibrio cholerae* ToxT requires binding to two centrally located DNA sites in an inverted repeat conformation

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Summary

The Gram-negative bacterium *Vibrio cholerae* is the infectious agent responsible for the disease Asiatic cholera. The genes required for *V. cholerae* virulence, such as those encoding the cholera toxin (CT) and toxin-coregulated pilus (TCP), are controlled by a cascade of transcriptional activators. Ultimately, the direct transcriptional activator of the majority of *V. cholerae* virulence genes is the AraC/XylS family member ToxT protein, the expression of which is activated by the ToxR and TcpP proteins. Previous studies have identified the DNA sites to which ToxT binds upstream of the *ctx* operon, encoding CT, and the *tcpA* operon, encoding, among other products, the major subunit of the TCP. These known ToxT binding sites are seemingly dissimilar in sequence other than being A/T rich. Further results suggested that *ctx* and *tcpA* each has a pair of ToxT binding sites arranged in a direct repeat orientation upstream of the core promoter elements. In this work, using both transcriptional *lacZ* fusions and *in vitro* copper-phenanthroline footprinting experiments, we have identified the ToxT binding sites between the divergently transcribed *acfA* and *acfD* genes, which encode components of the accessory colonization factor required for efficient intestinal colonization by *V. cholerae*. Our results indicate that ToxT binds to a pair of DNA sites between *acfA* and *acfD* in an inverted repeat orientation. Moreover, a mutational analysis of the ToxT binding sites indicates that both binding sites are required by ToxT for transcriptional activation of both *acfA* and *acfD*. Using copper-phenanthroline footprinting to assess the occupancy of ToxT on DNA having mutations in one of these binding sites, we found that protection by ToxT of the unaltered binding site was not affected, whereas protection by ToxT of the mutant binding site was significantly reduced in the region of the mutations. The results of further footprinting experiments using DNA templates having +5 bp and +10 bp insertions between the two ToxT binding sites indicate that both binding sites are occupied by ToxT regardless of their positions relative to each other. Based on these results, we propose that ToxT binds independently to two DNA sites between *acfA* and *acfD* to activate transcription of both genes.

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Introduction

The severe diarrhoeal disease cholera is caused by the Gram-negative, comma-shaped bacterium *Vibrio cholerae*. *V. cholerae* is usually acquired by ingestion of contaminated food or water (Finkelstein, 1973). The primary factor required for cholera disease is the cholera toxin (CT), an AB toxin encoded by the *ctxAB* genes (Lonnroth and Holmgren, 1973; Gill, 1976). Cholera toxin enters the intestinal epithelium by binding, with its five B subunits, to ganglioside GM1, followed by transport of the one active A subunit into the host cell (Holmgren *et al.*, 1973). The A subunit activates adenylate cyclase, resulting in a large increase in cyclic AMP (cAMP) levels. This induces secretion of fluid into the lumen of the intestine to produce the voluminous watery diarrhoea ('rice water stool') that is characteristic of cholera disease (Field *et al.*, 1972). *ctxAB* are carried within the genome of a lysogenic filamentous bacteriophage, CTXΦ (Waldor and Mekalanos, 1996). Colonization of the intestine by *V. cholerae* requires the toxin-coregulated pilus (TCP), a type IV pilus encoded by genes within the *tcpA* operon, which is expressed under the same environmental conditions as CT (Taylor *et al.*, 1987; Peterson and Mekalanos, 1988). TCP is also the receptor for CTXΦ (Waldor and Mekalanos, 1996). The genes encoding the TCP are located on the large *V. cholerae* chromosome within the vibrio pathogenicity island (VPI) (Peterson and Mekalanos, 1988; Kaufman *et al.*, 1993; Ogierman *et al.*, 1993; Karaolis *et al.*, 1998).

Expression of CT and TCP is subject to control by a complex network of positive and negative transcriptional

regulators. The direct activator of transcription of the genes encoding both CT and TCP is ToxT, a 32 kDa cytoplasmic protein (DiRita *et al*., 1991; Higgins *et al*., 1992). Expression of *toxT* from its own promoter is initiated by the combined activity of two membrane-localized transcriptional activators, ToxR and TcpP (Hase and Mekalanos, 1998; Krukonis *et al*., 2000; Krukonis and DiRita, 2003). These proteins, along with their protein cofactors, ToxS and TcpH, respectively, bind to DNA upstream of *toxT* and activate *toxT* transcription. Further expression of *toxT* is then induced by ToxT itself through positive feedback, via its role in activating transcription of the *tcpA* operon, within which the *toxT* gene is located (Higgins and DiRita, 1994; Brown and Taylor, 1995; Yu and DiRita, 1999). Expression of the *tcpPH* operon, which is also located within the VPI, is positively regulated by the transcriptional activators AphA and AphB, and negatively regulated by the cAMP receptor protein (CRP) and PepA protein (Kovacikova and Skorupski, 1999; 2001; 2002a; Skorupski and Taylor, 1999; Behari *et al*., 2001). Also implicated in this cascade is HapR, whose activity is controlled by the quorum-sensing system, and which negatively regulates expression of *aphA* (Jobling and Holmes, 1997; Kovacikova and Skorupski, 2002b; Miller *et al*., 2002; Zhu *et al*., 2002; Kovacikova *et al*., 2003).

ToxT protein is a member of the AraC/XylS family of transcriptional regulators (Higgins *et al*., 1992). AraC/XylS family members have in common a conserved DNA binding and transcriptional activation domain of approximately 100 amino acids, which contains two helix–turn–helix motifs (Gallegos *et al*., 1997; Martin and Rosner, 2001; Tobes and Ramos, 2002). Crystal structures that include the conserved domain have been determined for two AraC/XylS family members, MarA and Rob (Rhee *et al*., 1998; Kwon *et al*., 2000). The crystal structures of MarA and Rob bound to DNA suggest that the modes of DNA binding utilized by these two proteins differ; the structure of MarA bound to DNA indicates that both helix–turn–helix motifs specifically interact with the major groove, whereas the structure of Rob bound to DNA indicates that only the more N-terminal helix–turn–helix interacts specifically with the major groove. ToxT carries the 100-amino-acid AraC/XylS domain as its C-terminal domain. However, ToxT also has a 176-amino-acid N-terminal domain of unknown function. Other members of the AraC/XylS family having a second domain typically use this domain for multimerization and/or effector binding (Gallegos *et al*., 1997; Martin and Rosner, 2001; Tobes and Ramos, 2002). Whether ToxT is monomeric, as are some AraC/XylS family members, or forms dimers or multimers in solution, as do other AraC/XylS family members, has not been determined. The stoichiometry of ToxT binding to DNA has also not been determined.

Previous studies on ToxT function have focused primarily on the role of ToxT in activating expression of *ctx* and *tcpA*. ToxT was found to have dual roles in activating expression of *ctx* (Yu and DiRita, 2002). First, ToxT acts as an anti-repressor of *ctx*. It does so by competing for binding at DNA sites upstream of *ctx* with H-NS protein, which has multiple binding sites located in this region and acts to reduce expression of *ctx* (Nye *et al*., 2000). Second, ToxT acts as a direct activator of *ctx* transcription, presumably by interacting with RNA polymerase. In contrast to its negative effect upon *ctx* expression, H-NS has a minimal effect upon *tcpA* expression (Nye *et al*., 2000; Yu and DiRita, 2002). Therefore, at *tcpA*, ToxT acts not as an anti-repressor but only as a direct activator of transcription. This is most probably through interaction between ToxT and the C-terminal domain(s) of the α-subunits of RNA polymerase (α-CTD) (Hulbert and Taylor, 2002; Yu and DiRita, 2002).

The ToxT binding sites upstream of the *ctx* and *tcpA* core promoter elements were identified using both genetic methods and DNase I footprinting experiments (Hulbert and Taylor, 2002; Yu and DiRita, 2002). The sequences of these ToxT binding sites are somewhat dissimilar from each other, and a consensus sequence is not evident. The notable common features found in both the *ctx* and *tcpA* ToxT binding sites are poly A or poly T tracts of five or more contiguous A or T nucleotides on one strand of the DNA. The ToxT binding sites upstream of *ctx* have seven of these tracts; the ToxT binding sites upstream of *tcpA* have three of these tracts. Mutational analyses of the ToxT binding sites located upstream of *tcpA* using either a genetic screen (Hulbert and Taylor, 2002) or a site-directed approach (J.H. Withey and V.J. DiRita, in preparation) indicated that the promoter-proximal and promoter-distal A/T tracts are required for ToxT-dependent transcription of *tcpA*. Mutations in the central A/T tract had little to no effect on ToxT-directed transcription of *tcpA*. These results are consistent with ToxT binding to two sites upstream of *tcpA* in a direct repeat orientation, with each site containing one A/T tract.

In addition to its role in activating expression of the *ctx* and *tcpA* operons, ToxT also activates expression of at least five other genes: *acfA*, *acfD*, *aldA*, *tagA* and *tcpI* (Peterson and Mekalanos, 1988; Parsot and Mekalanos, 1991; Parsot *et al*., 1991). All of these genes are located within the VPI. As was found for *ctx* and *tcpA*, poly A or poly T tracts are found upstream of each of these genes, and these are the only obvious similarities in the DNA sequences upstream of these genes, which presumably contain ToxT binding sites. *acfA* and *acfD* are divergently transcribed genes that encode components of the accessory colonization factor (ACF) (Peterson and Mekalanos, 1988). The exact role of the ACF in pathogenesis is unclear, but it is required by *V. cholerae* for efficient colo-

nization of the infant mouse small intestine. *aldA* and *tagA* are also divergently transcribed genes. *aldA* encodes an aldehyde dehydrogenase (Parsot and Mekalanos, 1991). *tagA* encodes a putative transmembrane protein (Harkey *et al.*, 1995). The roles, if any, of *aldA* and *tagA* in pathogenesis are unknown. *tcpI* encodes a putative methyl-accepting chemotaxis protein (Harkey *et al.*, 1994). Again, the role, if any, of *tcpI* in pathogenesis is unknown.

In the studies described in this report, we used a combination of mutational analysis and copper-phenanthroline footprinting experiments to identify sites located between *acfA* and *acfD* that are required for ToxT binding and transcriptional activation. These ToxT binding sites are arranged in an inverted repeat orientation; this is in contrast to the previously described ToxT binding sites at *tcpA* and *ctx*, which are in direct repeat orientations. However, in similarity to *ctx* and *tcpA*, each of the *acfA/acfD* ToxT binding sites contains one A/T tract. Occupancy of both ToxT binding sites is required for transcription of both *acfA* and *acfD*. Mutation of one of the two binding sites does not significantly affect occupancy by ToxT of the unaltered binding site. Furthermore, both sites remain occupied by ToxT even when the spacing between the two binding sites is altered by the insertion of 5 or 10 base pairs (bp). Based on these results and previous work on other members of the AraC/XylS family, we propose that one ToxT monomer binds independently to each of the two binding sites between *acfA* and *acfD*, and that both ToxT monomers are required to make contacts with the RNA polymerase α-CTDs to activate transcription of *acfA* and *acfD*.

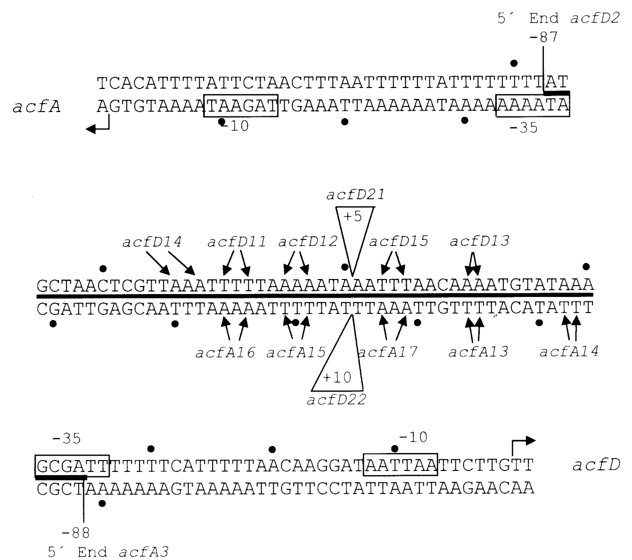

Fig. 1. Sequence of the *acfA–acfD* intergenic region. Both strands of the DNA are shown, with landmarks for *acfD* on the top strand and landmarks for *acfA* on the bottom strand. The start sites of transcription are shown by an arrow on the top strand for *acfD* and by an arrow on the bottom strand for *acfA*. The putative core –35 and –10 elements for each promoter are indicated by boxes. The region of overlap of the minimal ToxT-directed constructs for *acfA* and *acfD* is indicated by a thick line between the top and bottom strand sequences. The end points of the *acfD2* and *acfA4* constructs are also shown. The positions of mutations are indicated by arrows pointing from the mutant designation to the mutated base pairs. Each of the mutations changes an A/T base pair to a G/C base pair. As indicated, the *acfD11* and *acfA16* constructs have identical mutations at the same two base pairs, as do the *acfD12* and *acfA15*, *acfD15* and *acfA17* and *acfD13* and *acfA13* mutations. The position of the insertions in the *acfD21* and *acfD22* constructs is indicated by the triangles above and below the sequence. The +5 bp DNA sequence inserted in *acfD21* is GATCG on the top strand and the +10 bp DNA sequence inserted in *acfD22* is GATCGATCGA on the top strand.

Results

Identification of the minimal acfA *promoter region*

There is a near consensus –10 sequence (TATAAT) found upstream of both genes, the spacing of which is in good agreement with where primer extension experiments suggested each transcript initiates (data not shown). However, both genes have a degenerate putative –35 sequence at the appropriate position relative to the –10 element (Fig. 1). To determine the length of DNA upstream of the core promoter elements of *acfA* that is required for ToxT-directed transcription, we constructed nested *acfA::lacZ* fusions in which the 5′ end point of DNA upstream from the transcription start site for *acfA* varies from –146 to –66 (Fig. 2). These fusions were constructed in plasmid pTL61T, which has a promoterless *lacZ* gene downstream of a multiple cloning site (Linn and St Pierre, 1990). The effect of ToxT on expression of the fusions was determined by measuring β-galactosidase activity in wild-type and *toxT* mutant *V. cholerae* strains (O395 and VJ740 respectively; Champion *et al.*, 1997).

ToxT-dependent *acfA–lacZ* expression was observed with fragments harbouring DNA upstream of and including residue –88 (Figs 2 and 3). The next deletion in the series, *acfA4*, with its end point at –66, was inactive irrespective of the presence of ToxT. Deletion to –88 (*acfA3*) apparently removes a site that inhibits ToxT from activating the *acfA* promoter, as the *acfA3* construct expressed about two and a half times more ToxT-dependent β-galactosidase activity than either of the other two fusions with end points upstream of –88 (*acfA1* and *acfA2*). This was not accompanied by an elevated basal level of transcription (i.e. in the absence of ToxT) and thus the behaviour of this promoter is distinct from that previously observed with the *ctx* promoter (Yu and DiRita, 2002). In that analysis, deletion of specific upstream sequences led to increased basal transcription and a concomitantly increased level of ToxT-activated transcription (Yu and DiRita, 2002). This was attributed to the effects of the nucleoid-like protein H-NS, which is known to antagonize ToxT-dependent activation of the *ctx* promoter (Nye *et al.*, 2000; Yu and DiRita,

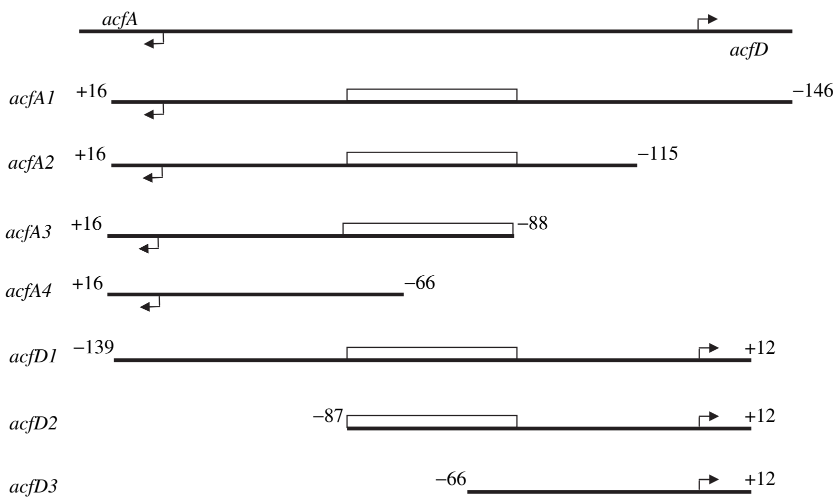

Fig. 2. Map of the nested *lacZ* fusion constructs. The black line at the top of the figure represents the entire *acfA–acfD* intergenic region. Arrows indicate transcriptional start sites. The fusion construct names are listed on the left side, and the size of the construct is represented by the length of the black line. The numbers on either side of the black line indicate the length of DNA present in the construct relative to the transcriptional start site. The open box indicates the region of overlap between the minimal *acfA::lacZ* and *acfD::lacZ* fusions from which ToxT is able to direct transcription.

2002). These results indicate that DNA sequence information required for ToxT-directed transcription of *acfA* resides within 88 nucleotides upstream of transcription initiation. The promoter is also subject to some measure of inhibition through sequence information between −115 and −88 (Fig. 3).

Mutational analysis of the acfA *promoter region*

The most significant similarities in sequence upstream of all the known ToxT-controlled genes are the presence of tracts of A or T nucleotides, so these tracts upstream of *acfA* were targeted for mutagenesis to assess their role in ToxT-directed transcription of *acfA*. Site-directed mutations were created in derivatives of the minimal ToxT-directed *acfA::lacZ* fusion, *acfA3*, which ends at position −88 (Fig. 1). The most dramatic effects were produced by changing the base pairs at −74 and −75 from A/T to G/C (*acfA13*) and by changing the base pairs at −54 and −56 from A/T to G/C (*acfA16*) (Fig. 3). In each case, the fusion was expressed to the same, low level in both the wild-type and Δ*toxT* backgrounds, suggesting that the base pairs at −74/−75 and −54/−56 are required for ToxT-directed transcription of *acfA*.

Two other mutations we introduced affected the basal (ToxT-independent) expression of the fusion alleles slightly, but we can nevertheless draw some conclusions about the requirements for the altered residues in ToxT-dependent activation. Allele *acfA17* exhibited a slightly higher baseline level of activation that was stimulated when ToxT was present (Fig. 3). The absolute level of ToxT-dependent expression was lower than the wild-type *acfA4* allele, and thus we conclude that the residues at −69 and −67 are important, but not essential, for ToxT to activate. The elevated activity in the absence of ToxT was not explored further. In *acfA15*, fusion activity in the *toxT* mutant background was somewhat lower than we saw with *acfA3* but, again, this allele was significantly stimulated in the presence of ToxT (Fig. 3). Activation of *acfA15* was the lowest of any of the mutated alleles that still

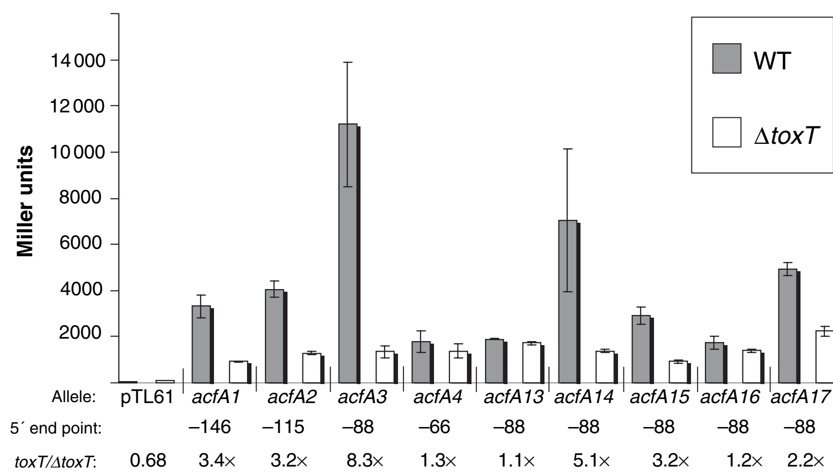

Fig. 3. Results of β-galactosidase assays on strains carrying *acfA::lacZ* fusions. The results of experiments performed with *V. cholerae* strains having wild-type ToxT (O395) are represented as grey bars, and the results of experiments performed with *V. cholerae* strains having a ToxT deletion (VJ740) are represented as white bars. 'Allele' indicates which *acfA::lacZ* fusion construct was used in the experiment, except for pTL61T, in which the empty vector was present in the indicated strains; '5′ end point' indicates the length of DNA upstream of the transcriptional start site that is present in the construct; and '*toxT/ΔtoxT*' indicates the fold difference between the mean β-galactosidase values for that construct measured in O395 and measured in VJ740. β-Galactosidase assays were performed in each strain a minimum of three times, and the values shown are the mean ± standard deviation.

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exhibited ToxT-directed transcription, and thus, as with *acfA17* we conclude that the A/T residues mutated in this allele (at –59 and –61) are important for, but not absolutely critical to, ToxT-dependent expression.

Finally, changing the base pairs at –82 and –83 from A/T to G/C, to create *acfA14*, had a minimal effect, if any, on transcription. As expected, mutations to A or T tracts located upstream of –88 had no effect on ToxT-directed transcription of *acfA* (data not shown).

Identification of the minimal acfD *promoter region*

We used a method similar to that described above for *acfA* to determine the length of DNA upstream of the core promoter elements of *acfD* that is required for ToxT-directed transcription. Nested *lacZ* fusions were constructed in pTL61T, and β-galactosidase levels were assayed in strains carrying these fusions and having either wild-type *toxT* or a deletion in *toxT*. This series extended from –139 bp upstream to –66 bp upstream of the transcription start site (Fig. 2). The first observation to note is that the ratio of ToxT-dependent to basal expression of *acfD* is much greater than that of *acfA*, reflecting primarily a lower basal promoter activity for *acfD* (Fig. 4).

The wild-type *toxT* strain carrying *acfD1* produced 24-fold more β-galactosidase activity than the Δ*toxT* strain carrying *acfD1* (Fig. 4). Deletion of the DNA sequence between –139 and –87 (*acfD2*) had no effect on ToxT-directed transcription of *acfD*. However, truncation of the DNA sequence to –66 (*acfD3*) abrogated ToxT-directed transcription of *acfD* (Fig. 4). Therefore, the sequence between –87 and –66 relative to the start of *acfD* transcription must contain determinants for ToxT-directed transcription of *acfD* (Fig. 1), and the region from –87 to +12 is sufficient for ToxT-directed transcription of *acfD*. The minimal *acfA4::lacZ* and *acfD2::lacZ* fusion constructs that exhibit ToxT-directed transcription of these genes overlap by 52 bp (Figs 1 and 2), suggesting that a single ToxT control region may exist between *acfA* and *acfD*.

Mutational analysis of the acfD *promoter region*

The A and T-rich sequences upstream of *acfD* were also targeted for mutation to determine their importance to ToxT-directed transcription of *acfD*. Site-directed mutations were created in derivatives of the minimal ToxT-directed *acfD::lacZ* fusion, *acfD2*, which ends at position –87 (Fig. 1) and is activated over 30-fold by ToxT. Mutations having the strongest negative effects on ToxT-directed transcription of *acfD2* were A/T to G/C alterations at –70/–68 (*acfD11*), –50/–49 (*acfD13*) and –74/–72 (*acfD14*). All of these mutant alleles expressed ToxT-dependent β-galactosidase at levels barely above background, and had *toxT/*Δ*toxT* ratios over 15 times lower than the wild-type allele. Thus, it is clear that these residues are essential for ToxT-dependent activation of *acfD*.

The other two mutant alleles we investigated in this series could still be activated by ToxT, but the level of activation was much lower than the *acfD2* allele. These are *acfD12*, with A/T to G/C changes at –65 and –63, and *acfD15*, with these changes at –57 and –55. *acfD15* in particular was still activated over eightfold by ToxT, but this level of activation is significantly lower than the 33-fold activation seen with *acfD2*. We conclude that these four residues are required for maximum ToxT-dependent expression, but that even without them ToxT remains capable of some degree of activation.

The mutations with the largest negative effect on ToxT-directed *acfD* transcription, *acfD11* and *acfD13*, are identical mutations to those in alleles *acfA16* and *acfA13*,

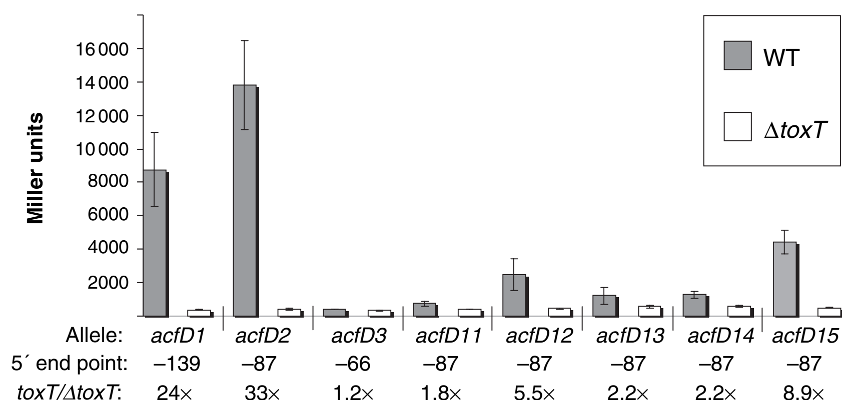

Fig. 4. Results of β-galactosidase assays on strains carrying *acfD::lacZ* fusions. The results of experiments performed with *V. cholerae* strains having wild-type ToxT (O395) are represented as grey bars, and the results of experiments performed with *V. cholerae* strains having a *toxT* deletion (VJ740) are represented as white bars. 'Allele' indicates which *acfD::lacZ* fusion construct was used in the experiment; '5′ end point' indicates the length of DNA upstream of the transcriptional start site that is present in the construct; and '*toxT/*Δ*toxT*' indicates the fold difference between the mean β-galactosidase values for that construct measured in O395 and measured in VJ740. β-Galactosidase assays were performed in each strain a minimum of three times, and the values shown are the mean ± standard deviation.

respectively (Fig. 1), which were the most severely affected for ToxT-directed transcription (Figs 3 and 4). This suggests that ToxT activates transcription of both genes from the same central control region.

Identification of the ToxT binding sites between acfA *and* acfD *by copper-phenanthroline footprinting*

The genetic experiments described above allowed us to determine regions between *acfA* and *acfD* that are required for ToxT-directed transcription. Electrophoretic mobility shift analysis (EMSA) confirmed that purified ToxT binds specifically to the *acfA–acfD* intergenic DNA (data not shown) but did not provide any information regarding the stoichiometry of binding. This is because, as we have previously shown, ToxT–DNA complexes enter the gel poorly, and we are not able to resolve distinct complexes of bound monomers versus bound dimers (e.g. Yu and DiRita, 2002).

To more precisely identify the DNA sequence bound by ToxT we used DNA footprinting. Standard DNase I footprinting gave unclear results (data not shown), presumably because the DNA sequence between *acfA* and *acfD* has abundant A and T nucleotides, including numerous poly A tracts, and these are poorly cleaved by DNase I due to narrowing of the minor groove (Nelson *et al.*, 1987; Urbach and Dervan, 2001). Instead, we used the copper-phenanthroline footprinting technique, in which chemical digestion of the DNA was performed after electrophoretic separation of the H_6-ToxT/DNA complexes and free DNA. The chemically digested protein/DNA complexes and free DNA were then eluted from the EMSA gel and run side by side on a sequencing gel beside a sequencing ladder to assess where H_6-ToxT had prevented chemical cleavage of the DNA, indicating a region of specific binding. As shown in Fig. 5, we observed a single region of protection between *acfA* and *acfD* conferred by H_6-ToxT, bracketed by hypersensitive sites at positions –49 and –77 relative to the *acfA* transcription start site or –47 and –75 relative to the *acfD* transcription start site. This protection is in the same DNA region we identified as being required for ToxT-directed transcription of both *acfA* and *acfD* in the *lacZ* fusion experiments described above. More specifically, the 5′ end points of the *acfA4::lacZ* and *acfD3::lacZ* fusion constructs, which do not exhibit ToxT-directed transcription, are within the DNA sequence protected by ToxT in these experiments, indicating that the failure of ToxT to activate transcription of these constructs results from disruption of the ToxT/DNA binding region. The strongest protection was seen in the area around the two A/T tracts, mutation of which completely abrogated activation of transcription by ToxT. The areas between the A/T tracts showed less complete protection (Fig. 5).

The DNA sequence between *acfA* and *acfD* that is

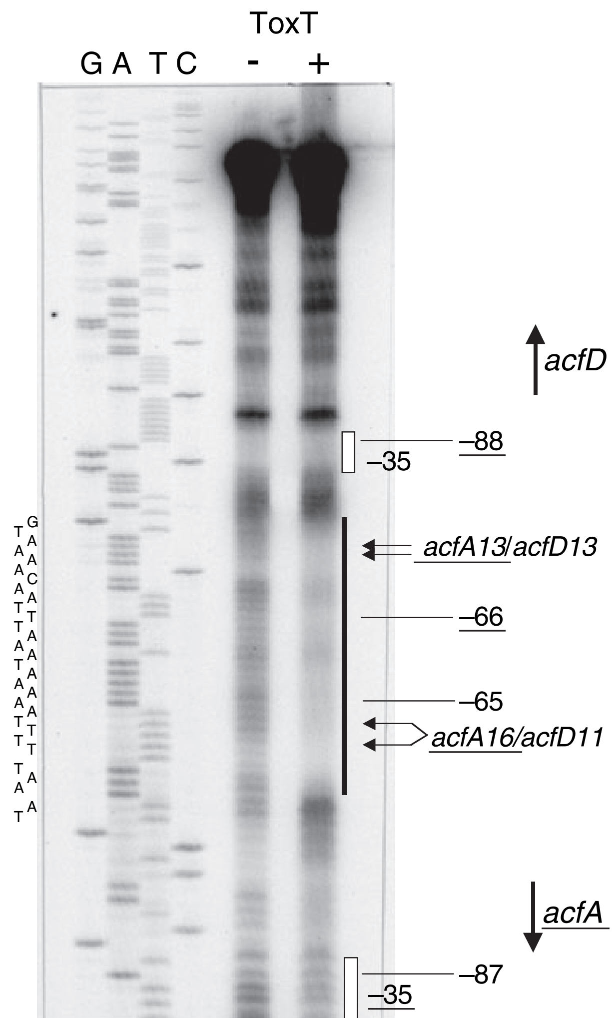

Fig. 5. Copper-phenanthroline footprinting of H_6-ToxT in the *acfA–acfD* intergenic region. The lane having DNA cleaved by copper-phenanthroline in the absence of H_6-ToxT is indicated by the '–' symbol and the lane having H_6-ToxT/DNA complexes cleaved by copper-phenanthroline is indicated by the '+' symbol. The numbers to the right of the autoradiograph indicate the distances upstream of the transcriptional start sites of *acfA* and *acfD*, with numbers applying to *acfD* in standard type and numbers applying to *acfA* underlined. The –35 core promoter elements are represented by empty boxes. The vertical line indicates the region of protection by H_6-ToxT. The sequence of the inverted repeat region depicted in Fig. 6A is shown beside the sequencing lanes. The locations of mutations that strongly affect ToxT-directed transcription of *acfA* and *acfD* are shown by arrows extending from the mutant designation.

protected from chemical cleavage by ToxT contains a degenerate inverted repeat sequence, with 9 out of 13 nucleotides in each side of the repeat having the same identity (asterisks in Fig. 6A). The 5′ portion of each side of the repeat sequence contains a T tract, and the 3′ portion is A/T rich. Mutations to the 5′ T tract of either side of the repeat sequence abrogated ToxT-dependent transcription of both genes (*acfA13*, *acfA16*, *acfD11*, *acfD13*). Comparison of these ToxT binding sites to the previously

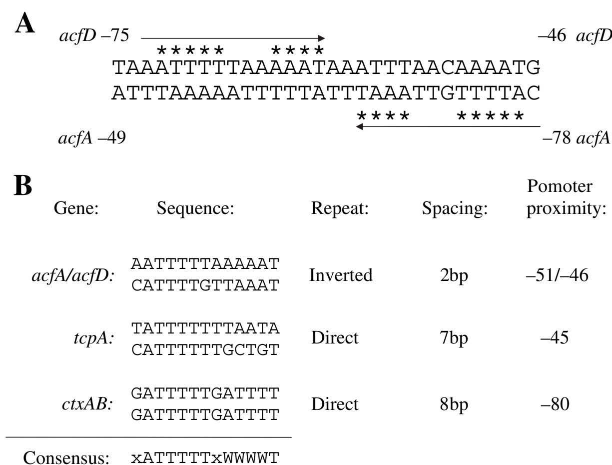

Fig. 6. A. Sequence of the inverted repeat between *acfA* and *acfD*. The nucleotides that are identical in the two binding sites are indicated by asterisks above or below the sequence. Arrows represent the length and orientation of the putative binding sites.
B. Comparison of the *acfA/acfD* ToxT binding sites to the ToxT binding sites previously identified upstream of *tcpA* and *ctx*. 'Spacing' indicates the distance between the two ToxT binding sites. 'Promoter proximity' indicates the promoter-proximal end of the nearest ToxT binding site relative to the start site of transcription. The consensus sequence is designated based on at least five out of the six binding sites having the indicated nucleotide at the indicated position. W signifies that the nucleotide can be either A or T.

identified ToxT binding regions upstream of *ctx* and *tcpA* shows conservation of the T tract in the 5′ half of the binding site (Fig. 6B). The 3′ portion of the binding sites are A/T rich, but have no specific sequence conservation. The most significant difference between the *acfA* and *acfD* ToxT binding sites and the *ctx* and *tcpA* ToxT binding sites is that the former are arranged in an inverted repeat orientation, whereas the latter are arranged in a direct repeat orientation (J.H. Withey and V.J. DiRita, in preparation).

Mutations that prevent ToxT-directed transcription reduce specific binding of ToxT only to the mutated DNA binding site

The results described above suggest that ToxT binds to two DNA sites in an inverted repeat configuration between *acfA* and *acfD*, and that occupancy of both of these DNA sites is required for ToxT-directed transcription of both *acfA* and *acfD*. We next asked how the occupancy of the putative ToxT binding sites is changed when one of the sites has mutations that prevent ToxT from activating transcription. EMSA experiments indicated that ToxT still binds specifically to the mutated DNA sites (data not shown). To further address this question, we performed copper-phenanthroline footprinting experiments on complexes of H_6-ToxT and DNA from mutants that are defective for ToxT-directed transcription of *acfA* and/or *acfD*. These mutants have alterations in either one or the other of the two putative ToxT DNA sites located between *acfA* and *acfD*. DNA from the *acfA16* construct, which has mutations in the T tract of the *acfA*-proximal ToxT binding site (and which has mutations identical to those in the *acfD11* construct; Fig. 1), shows reduced protection by ToxT from chemical cleavage in the mutated binding site surrounding the location of the mutations (arrows in Fig. 7). However, the other ToxT binding site shows no apparent reduction in protection by ToxT. Likewise, DNA from the *acfA17* construct, which has mutations in the 3′ A/T-rich portion of the *acfA*-distal ToxT binding site (and which has mutations identical to those in the *acfD15* construct; Fig. 1), shows reduced protection from chemical cleavage by ToxT in the mutant binding site (Fig. 7). The unaltered ToxT binding site, however, shows no apparent reduction in protection by ToxT. This mutant is particularly notable because it reduces ToxT-directed transcription of *acfA* (*acfA17*) but has little effect on ToxT-directed transcription of *acfD* (*acfD15*). These results strongly suggest that ToxT has two DNA binding sites between *acfA* and *acfD*.

Insertions between the two ToxT binding sites do not abrogate ToxT binding to either site

The above results indicating that mutations to one ToxT binding site do not affect protection by ToxT of the unaltered binding site raise the question of whether ToxT binds independently to the two sites between *acfA* and *acfD*. This question is made more compelling by our observations that ToxT uses a variety of binding site configurations to activate transcription of different genes. For *acfA* and *acfD*, as described above, and for *tagA* (J.H. Withey and V.J. DiRita, in preparation), ToxT binds to two sites in inverted repeat configurations. For *ctx* and *tcpA*, ToxT binds to two sites in direct repeat configurations (J.H. Withey and V.J. DiRita, in preparation). And for *aldA*, ToxT binds to a single site (J.H. Withey and V.J. DiRita, in preparation).

To address the question of whether ToxT binds independently to its two sites between *acfA* and *acfD*, we created derivatives of *acfD2* having insertions of 5 bp or 10 bp between the two ToxT binding sites, creating the *acfD21* and *acfD22* constructs, respectively (Fig. 1), and performed copper-phenanthroline footprinting with H_6-ToxT on DNA amplified from these templates. We would predict that footprinting to one or both of the ToxT sites would be lost in the presence of a 5 bp insertion between the sites if interactions between the ToxT molecules bound to the two sites are required for binding. Because these constructs are derivatives of *acfD2*, they show footprints on the opposite DNA strand from those presented earlier in this report using derivatives of *acfA3* (Figs 5 and 7).

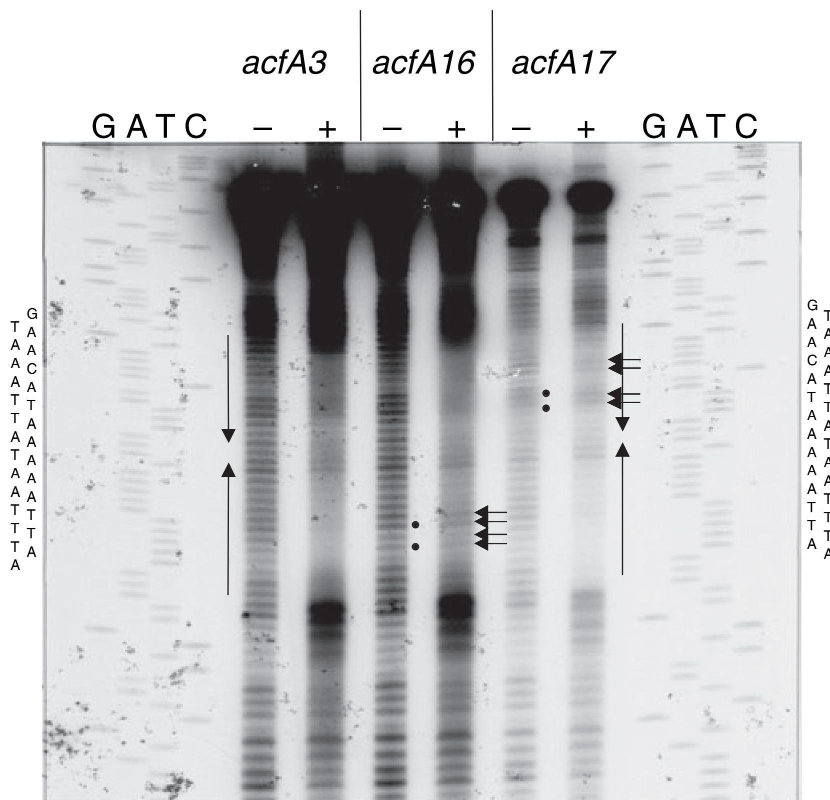

Fig. 7. Copper-phenanthroline footprinting of H_6-ToxT on *acfA* mutants defective in ToxT-directed transcription. The lanes having DNA cleaved by copper-phenanthroline in the absence of H_6-ToxT are indicated by the '–' symbol and the lanes having H_6-ToxT/DNA complexes cleaved by copper-phenanthroline are indicated by the '+' symbol. The template construct from which the labelled DNA visible in the gel was amplified by PCR is shown above each pair of lanes. The sequence of the top strand of the inverted repeats depicted in Fig. 6A is indicated beside the sequencing lanes. The long arrows represent the putative ToxT binding sites. The black dots beside the free DNA lanes indicate where mutations are located. The short arrows denote nucleotides at which protection by H_6-ToxT is decreased in the mutated binding sites relative to the wild-type binding sites.

Clear protection was conferred by H_6-ToxT in the area of the binding sites previously identified using *acfA3* derivatives (Fig. 8, left). However, the hypersensitive sites produced by H_6-ToxT in footprints on *acfA3* and derivatives (Figs 5 and 7) were not observed in footprints on *acfD2* and derivatives. The 3′ end of the *acfD*-proximal binding site was also protected to a larger degree by H_6-ToxT in *acfD2* and derivatives than in *acfA3* and derivatives. The regions of DNA exhibiting H_6-ToxT-mediated protection were otherwise identical in footprints of both strands.

Clear protection was also conferred by H_6-ToxT to *acfD21* DNA, which has 5 bp inserted between the two binding sites (Fig. 8, middle). Significantly, both binding sites were protected by H_6-ToxT, despite the fact that these sites have been rotated approximately one half turn of the DNA helix relative to their wild-type spacing. This strongly suggests that the ToxT molecules bound to the two binding sites are not interacting with each other. Finally, protection was also conferred by H_6-ToxT to *acfD22* DNA, which has 10 bp inserted between the two binding sites (Fig. 8, right). To assess the effects of insertions between the binding sites on transcriptional activation by ToxT, β-galactosidase assays were performed on *acfD21::lacZ* and *acfD22::lacZ* fusions. Neither of these constructs was able to support ToxT-directed transcription (Fig. 9). This finding suggests that, although both of the ToxT binding sites are occupied in both the *acfA21* and *acfA22* constructs, this occupancy is not sufficient to allow ToxT to activate transcription. Therefore, the spacing of the binding sites relative to the promoter is an important factor in ToxT-directed transcription.

Discussion

The experiments described in this report were designed to determine the DNA sequence requirements for ToxT-directed transcription of *acfA* and *acfD*, genes that have poorly understood roles in the pathogenesis of *V. cholerae*, but were previously shown to be part of the ToxR regulon (Peterson and Mekalanos, 1988; Parsot and Mekalanos, 1992). Using nested *lacZ* fusions, we identified the minimal DNA sequences upstream of both *acfA* and *acfD* required for ToxT-directed transcription of the respective genes. The minimal ToxT-directed DNA constructs, *acfA3* and *acfD2*, overlap by 52 bp (Figs 1 and 2). This overlap of the DNA sequences required for ToxT-directed transcription of *acfA* and *acfD* suggests that there may be a single control region between the two genes utilized by ToxT to activate transcription of both genes.

Mutational analyses of the minimal ToxT-directed DNA constructs for *acfA* and *acfD* identified some of the specific sequence requirements for ToxT-directed transcription of these genes. Examination of the DNA sequences upstream of all the genes known to be within the ToxT

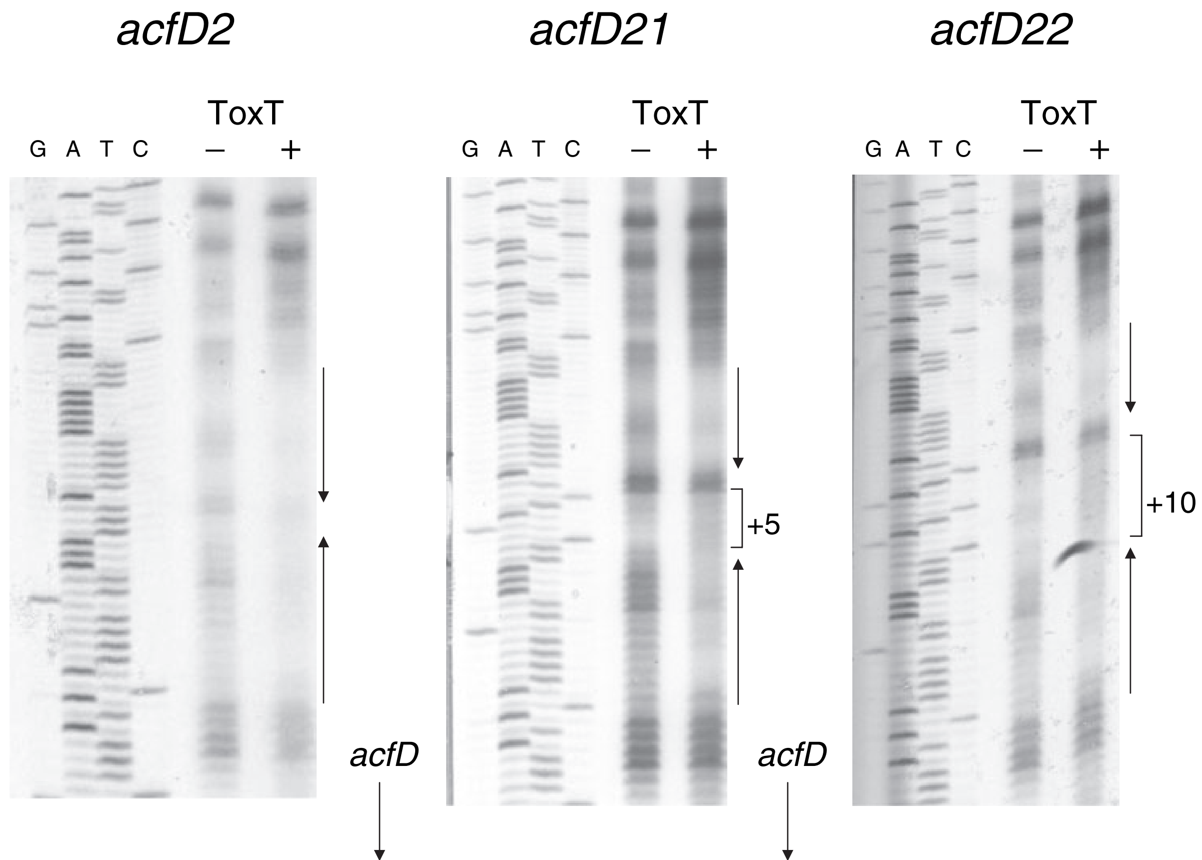

Fig. 8. Copper-phenanthroline footprinting of H_6-ToxT on *acfD* constructs having insertions between the two ToxT binding sites. The lanes having DNA cleaved by copper-phenanthroline in the absence of H_6-ToxT are indicated by the '–' symbol and the lanes having H_6-ToxT/DNA complexes cleaved by copper-phenanthroline are indicated by the '+' symbol. The template construct from which the labelled DNA visible in the gel was amplified by PCR is shown above each pair of lanes. The long arrows represent the putative ToxT binding sites. The brackets beside the bound DNA lanes indicate where insertions between the binding sites are located.

regulon yields no obvious consensus DNA binding sequence. However, tracts of A and T nucleotides are evident upstream of the core promoter elements of each gene. The results of our mutational analyses of some of the A and T-rich sequences between *acfA* and *acfD* determined some of the DNA sequence that is specifically required for ToxT-directed transcription of these two genes. Identical mutations in separate *acfA::lacZ* and *acfD::lacZ* fusions had similar effects on β-galactosidase expression from both fusions. For example, the *acfA16* and *acfD11* constructs have A/T to G/C changes to two base pairs at the same positions (Fig. 1). Comparison of β-galactosidase production in strains carrying the *acfA16::lacZ* and *acfD11::lacZ* mutant constructs indicates little to no ToxT-dependent transcription from the mutant constructs, strongly suggesting that the base pairs targeted for mutation are required for ToxT-directed transcription of both *acfA* and *acfD*. Similarly, the *acfA13::lacZ* and *acfD13::lacZ* constructs, which have identical mutations, both allowed no ToxT-directed tran-

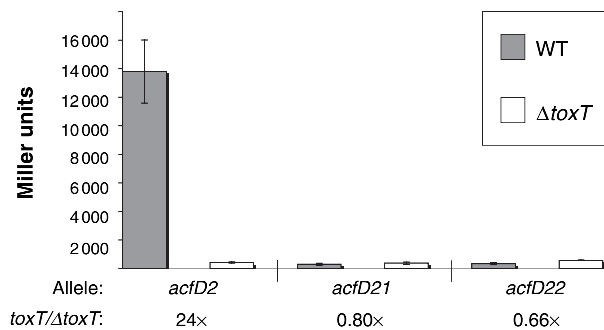

Fig. 9. Results of β-galactosidase assays on strains carrying *acfD::lacZ* fusions with +5 bp or +10 bp insertions between the ToxT binding sites. The results of experiments performed with *V. cholerae* strains having wild-type ToxT (O395) are represented as grey bars, and the results of experiments performed with *V. cholerae* strains having a ToxT deletion (VJ740) are represented as white bars. 'Allele' indicates which *acfD::lacZ* fusion construct was used in the experiment and '*toxT/ΔtoxT*' indicates the fold difference between the mean β-galactosidase values for that construct measured in O395 and measured in VJ740. β-Galactosidase assays were performed in each strain a minimum of three times, and the values shown are the mean ± standard deviation.

scription. Constructs carrying the identical *acfA15* and *acfD12* mutations both exhibited reduced, but still significant, levels of activation by ToxT. Again, these results strongly suggest that ToxT is acting at a single location between *acfA* and *acfD* to activate transcription of both genes.

The only mutations that caused significant differences in the degrees of activation by ToxT were the identical *acfA17/acfD15* mutations. The *acfA17::lacZ* construct produced only 2.2-fold higher levels of β-galactosidase in the wild-type *toxT* strain than in the Δ*toxT* strain, whereas the *acfD15::lacZ* construct produced 8.9-fold higher levels of β-galactosidase in the wild-type *toxT* strain than in the Δ*toxT* strain. The *acfA17::lacZ* construct did have a considerably higher basal level of transcription than the other *acfA::lacZ* constructs, which may or may not have any bearing on this comparison. If the β-galactosidase levels of these mutants in the presence of ToxT are compared with the β-galactosidase levels in the wild-type constructs, as the relative percentage of gene expression, the differences are less dramatic. Using this analysis, the *acfA17* construct had 44% relative gene expression, and the *acfD15* construct had 32% relative gene expression, suggesting that the identical mutations have similar effects on expression of both *acfA* and *acfD*. However, it is possible that the base pairs that are mutated in these constructs have different roles in activation of *acfA* and *acfD*.

Copper-phenanthroline footprinting studies confirmed that ToxT binds to a single location between *acfA* and *acfD*. In these studies, a 28 bp segment of DNA at a central location between *acfA* and *acfD* was strongly protected by ToxT, with complete protection seen in the regions of the A/T tracts that are critical for ToxT-directed transcription of both *acfA* and *acfD*. The overall region of protection is consistent with our results from the *lacZ* fusion experiments. The 5′ end points of the *acfA4* and *acfD3* constructs, from which ToxT is unable to activate transcription, are within the region protected by ToxT in the footprinting experiments. The obvious explanation for this is that ToxT cannot activate transcription from a construct having an incomplete set of DNA binding sites. The 5′ end points of the minimal *acfA3* and *acfD2* constructs, from which ToxT is able to activate transcription, are well beyond the region protected by ToxT in the footprinting experiments, as would be expected.

Examination of the DNA sequence that shows protection by ToxT in the footprinting experiments and is required for ToxT-directed transcription of *acfA* and *acfD* suggests that ToxT binds to two binding sites oriented as an inverted repeat between the two genes (Fig. 6A). This is unexpected considering that the previously identified ToxT binding sites upstream of *ctx* and *tcpA* are most probably oriented as direct repeats (J.H. Withey and V.J. DiRita, in preparation). Moreover, as described above, mutations in either of the putative ToxT binding sites between *acfA* and *acfD*, and particularly in the A/T tracts, have similar effects on transcription of both genes. We believe that the 13 bp length of the putative ToxT binding sites is consistent with each of these sites being bound by a single ToxT monomer, based on the lengths of DNA binding sites identified for other proteins in the AraC/XylS family. For example, the related monomeric MarA, SoxS and Rob proteins from *Escherichia coli* all bind to a degenerate 19 bp DNA sequence (Martin *et al*., 1999; Griffith and Wolf, 2001), and the dimeric RhaS and AraC proteins each bind to a pair of 17 bp binding sites (Egan and Schleif, 1994; Schleif, 2000). The dimeric AdpA protein from *Streptomyces griseus* binds to a pair of 10 bp binding sites (Yamazaki *et al*., 2004). AraC/XylS family proteins have a pair of helix–turn–helix motifs within their DNA binding domains, and thus have the capability to bind to two consecutive major grooves per protein monomer. The lengths of the DNA sequences that are protected by ToxT in footprinting experiments both shown in this report for the *acfA–acfD* promoter regions and shown previously for the *tcpA* and *ctx* promoter regions (Yu and DiRita, 2002) are most probably too large to be occupied by a single ToxT monomer. The observation that there are two conserved A/T tracts found upstream of *acfA*, *acfD* and *tcpA* (J.H. Withey and V.J. DiRita, in preparation), both of which are required for activation of these genes by ToxT, and the observation that these tracts are in a direct repeat orientation upstream of *tcpA* and an inverted repeat orientation upstream of *acfA* and *acfD*, also make the occupancy of this region by a single ToxT monomer unlikely. Therefore, we propose that one ToxT monomer binds to each of the two binding sites. This idea is further supported by our observation that only a single ToxT binding site is used by ToxT for activation of transcription of *aldA* (J.H. Withey and V.J. DiRita, in preparation).

Further copper-phenanthroline footprinting studies that assessed the occupancy of the two putative ToxT DNA binding sites when one site carries mutations showed reductions in the protection conferred by ToxT in the region of the mutations. Footprinting experiments using DNA from the *acfA16* (*acfD11*) construct, which has a pair of mutations that abrogates ToxT-directed transcription of *acfA*, showed reduced protection by ToxT when compared with the protection conferred by ToxT to DNA from the wild-type *acfA3* construct (Fig. 7). However, this reduced protection by ToxT was only evident in the area of the mutations; the unaltered binding site showed no obvious reduction in protection by ToxT. Similarly, experiments using DNA from the *acfA17* (*acfD15*) construct, which has a pair of mutations in the 3′ portion of the other putative ToxT binding site that reduces ToxT-directed tran-

scription of *acfA* and *acfD* showed reduced protection by ToxT in the region of the mutations. Again, the unaltered binding site showed no obvious reduction in protection by ToxT. This loss of protection is consistent with the idea that occupancy of both binding sites by ToxT, or at least of the regions of the binding sites containing the conserved A/T tracts, is required for activation of transcription by ToxT.

The finding that mutations to one ToxT binding site affect occupancy by ToxT of that site but not occupancy of the unaltered site, together with our observations that ToxT uses a variety of binding site configurations, including inverted repeats, direct repeats and a single binding site, to activate transcription of different genes, raised the question of whether interactions between bound ToxT molecules are required for transcriptional activation. If ToxT is indeed able to bind to pairs of DNA sites having both direct and inverted repeat orientations, as we propose, the protein must be remarkably flexible to form or maintain protein–protein interactions between ToxT monomers in these different orientations. To address this question we altered the spacing between the two *acfA/acfD* ToxT binding sites by +5 bp and +10 bp and asked whether ToxT would footprint the altered DNA. If ToxT requires interactions between the monomers bound at both sites, or binds to DNA as a dimer, rotating the binding sites one half turn of the helix relative to each other should disrupt any interactions between ToxT molecules and the footprinting of one or both ToxT sites should be affected. Our results strongly suggest that ToxT is able to footprint both binding sites regardless of their positions relative to each other. Therefore, we propose that ToxT binds as independent monomers to the two sites between *acfA* and *acfD*.

The consensus DNA sequence for ToxT binding that we have proposed in this work has remarkably little character, other than the presence of a T tract at the 5′ end of the site and preference for A/T-rich sequence at the 3′ end of the site. This degeneracy is not unusual for AraC/XylS family protein DNA binding sites (Martin *et al*., 1999; Thomas and Collins, 1999; Ibarra *et al*., 2003). Studies that have identified the ToxT binding sites upstream of several other genes upon which ToxT acts (J.H. Withey and V.J. DiRita, in preparation) are consistent with the consensus DNA sequence described here. Clearly there must be other contextual cues for ToxT to recognize appropriate binding sites, for a search of only the VPI using this consensus sequence generates dozens of potential ToxT sites, the vast majority of which are not utilized. We propose that the additional contextual information is mediated by interactions between ToxT and RNA polymerase. This may occur by a mechanism known as DNA scanning or pre-recruitment that has been proposed for other AraC family members having degenerate DNA binding sites (Griffith *et al*., 2002; Martin *et al*., 2002). According to this model, interaction between the activator protein and RNA polymerase occurs before DNA binding. The extra binding specificity conferred by the RNA polymerase/activator complex permits identification of the appropriate binding sites for that particular activator. In the absence of an adjacent promoter, ToxT would not maintain occupancy of its binding site, and would instead diffuse away with RNA polymerase in search of an appropriate binding site. Previous studies using the *tcpA* promoter have shown that ToxT most probably interacts with the α-CTD of RNA polymerase (Hulbert and Taylor, 2002; Yu and DiRita, 2002). The spacing of ToxT binding sites described here, relative to the core promoter elements, would also be consistent with ToxT interacting with α-CTD of RNA polymerase as at a class I promoter. Another possibility, provided that ToxT monomers bound to different DNA sites interact with each other, is that only DNA sites with the appropriate spacing(s) and/or orientation(s) can maintain occupancy by ToxT.

Regulation of transcription from divergent promoters by centrally located proteins is quite common in bacteria (Raibaud *et al*., 1989; Marques *et al*., 1998; Rhee *et al*., 1999; Thomas and Collins, 1999; Browning *et al*., 2002; Recchi *et al*., 2003). One particularly relevant example is the *Mycobacterium tuberculosis* Rv1395 activator protein (Recchi *et al*., 2003), which is also an AraC/XylS family member, although the existence of multiple promoters for both of the divergent genes it regulates, *Rv1394c* and *Rv1395*, makes that example considerably more complicated than what we observed for *acfA* and *acfD*, each of which has a single promoter. Another AraC family member, UreR, activates transcription of the divergent *ureD* and *ureR* genes (D'Orazio *et al*., 1996; Thomas and Collins, 1999). However, control of these genes is mediated from independent UreR binding sites, so it is unlike what we observed for *acfA* and *acfD*, which share a pair of ToxT binding sites. Another well-described protein that can coordinate control of divergently transcribed genes is CRP (El-Robh and Busby, 2002). Divergent promoters that share a central CRP binding site were shown to be independently activated by CRP (El-Robh and Busby, 2002). In that work CRP was acting at class II promoters, at which the regulatory DNA site overlaps the –35 element, for both of the divergently transcribed genes. In contrast, ToxT presumably acts at class I promoters, at which the regulatory DNA site is upstream of the –35 element, to activate both *acfA* and *acfD*. Finally, ToxT itself also activates transcription of the divergent *aldA* and *tagA* promoters (Parsot and Mekalanos, 1991; Parsot *et al*., 1991); however, these promoters have independent control regions and there is no overlap between the minimal ToxT-directed *aldA* and *tagA* promoter constructs (J.H. Withey and V.J. DiRita, in preparation). Because *acfA* and *acfD*

both encode components of the ACF, it is logical that they would be co-ordinately regulated, whereas *aldA* and *tagA* encode an aldehyde dehydrogenase and a putative lipoprotein, respectively, the co-ordinate expression of which may be undesirable.

A previous study on the activation of *acfA* and *acfD* transcription indicated that DNA topology is an important factor in the regulation of these genes in *V. cholerae* (Parsot and Mekalanos, 1992). The results of this previous work suggested that the *acfA–acfD* region carried on a plasmid in *V. cholerae* did not show significant transcriptional activation of either gene under inducing conditions, whereas *acfA–acfD* constructs carried on the bacterial chromosome did show transcriptional activation of both genes under inducing conditions. The most likely explanation for the difference in results between the previous work and our current work is that Parsot and Mekalanos compared expression of their fusions in strains grown under different environmental conditions [37°C, Luria–Broth (LB) at pH 8.4 as non-inducing; 30°C, LB at pH 6.5 as inducing], whereas we compared expression of our fusions in strains having wild-type *toxT* or a deletion of the region encoding a helix–turn–helix in *toxT* (VJ740); in all of our experiments the bacteria were grown at 30°C in LB at pH 6.5. Similar experiments we performed in a strain having a complete, in-frame deletion of *toxT* had results very similar to those obtained using VJ740 (data not shown). Because the experiments described here are geared towards examining only the role of ToxT in activation of *acfA* and *acfD*, we believe our experimental system has the fewest variables, and thus is more appropriate for pinpointing ToxT function. However, it is certainly plausible that DNA topology differs when bacteria are grown under different environmental conditions, and this difference in topology could affect gene expression.

To summarize, we have identified the ToxT binding sites located between *acfA* and *acfD* required for transcriptional activation of both genes. Two ToxT binding sites arranged in an inverted repeat orientation are located between *acfA* and *acfD* and both binding sites are required for ToxT-directed transcription of both *acfA* and *acfD*. Mutations to one ToxT binding site do not significantly affect occupancy of the second binding site, and the sizes of the binding sites are consistent with one ToxT monomer binding to each site. DNA insertions between the binding sites did not affect occupancy by ToxT of either site, suggesting that ToxT binds independently to the two sites. However, these insertions did abrogate activation of transcription by ToxT, suggesting that spacing of the binding sites relative to the promoter is an important factor in activation. Both ToxT monomers probably contact RNA polymerase, presumably via the α-CTDs, bound to either the *acfA* or *acfD* promoter.

Experimental procedures

Bacterial strains and plasmids

Strains used in this study are listed in Table 1. Strains were grown at 37°C in LB medium for overnight cultures, and at 30°C in LB adjusted to a starting pH of 6.5 (inducing conditions) for use in β-galactosidase assays. Strains were maintained at −70°C in LB + 20% glycerol. Antibiotics were used at the following concentrations: ampicillin, 100 $\mu g\ ml^{-1}$; streptomycin, 100 $\mu g\ ml^{-1}$. Plasmids were introduced into *V. cholerae* strains by electroporation using a Bio-Rad *E. coli* Pulser.

DNA manipulations

Plasmids were purified using the Qiagen Spin Miniprep or Plasmid Midi kits. Polymerase chain reaction (PCR) was performed using Taq DNA polymerase from Roche as specified by the manufacturer. Restriction enzymes were purchased from New England Biolabs and used as specified by the manufacturer.

Plasmid construction

The *acfA* and *acfD* nested *lacZ* fusions were constructed by PCR of the appropriate region using fresh O395 colonies as template. PCR products were cloned between the *Xba*I and *Hin*dIII sites of pTL61T (Linn and St Pierre, 1990). Site-directed mutations were created using the splicing by overlap extension technique (SOE) (Horton *et al*., 1989; 1993), after which inserts having the desired mutations were cloned between the *Xba*I and *Hin*dIII sites of pTL61T. The nucleotide sequences of all plasmid constructs were confirmed by DNA sequencing at the University of Michigan Sequencing Core.

β-Galactosidase assays

For β-galactosidase assays, *V. cholerae* strains were grown overnight at 37°C, subcultured at a 1:40 dilution into fresh LB, pH 6.5, and grown for 3 h at 30°C. Bacteria were then placed on ice and chloramphenicol was added to 0.5 mg ml^{-1}. Assays were performed according to the method of Miller (1972).

Copper-phenanthroline footprinting

EMSA was performed as previously described (Yu and DiRita, 2002), except the DNA probe used was a PCR product made using one ^{32}P-end-labelled primer and one unlabelled primer. The amount of H_6-ToxT used was determined empirically to be the amount required to shift approximately 50% of the labelled DNA. Plasmids used in the β-galactosidase assays were used as PCR templates as indicated in the text and Table 1. The PCR template used to produce the DNA for footprinting in Fig. 7 was pJW63 (*acfA1*). After EMSA, the procedure used was that of Papavassiliou (1994). Briefly, the gel was soaked in 200 ml of 10 mM Tris-HCl, pH 8 in a glass tray. One millilitre of 40 mM 1,10-Phenanthroline (Sigma) was mixed with 1 ml of 9 mM $CuSO_4$ (Sigma) for 1 min, then diluted with 18 ml dH_2O. This was added to the gel tray and

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Table 1. Strains used in this study.

Strain	Plasmid	Relevant genotype	Parent strain	Source
O395		Str^R		Laboratory collection
VJ740		*ΔtoxT*	O395	Champion *et al.* (1997)
JW21	pTL61T	Amp^R	O395	This work
JW22	pTL61T	Amp^R	VJ740	This work
JW40	pJW63	*acfA1::lacZ*	O395	This work
JW41	pJW64	*acfA2::lacZ*	O395	This work
JW44	pJW63	*acfA1::lacZ,ΔtoxT*	VJ740	This work
JW45	pJW64	*acfA2::lacZ,ΔtoxT*	VJ740	This work
JW64	pJW75	*acfA4::lacZ*	O395	This work
JW66	pJW75	*acfA4::lacZ,ΔtoxT*	VJ740	This work
JW86	pJW81	*acfA3::lacZ*	O395	This work
JW88	pJW83	*acfD1::lacZ*	O395	This work
JW89	pJW84	*acfD2::lacZ*	O395	This work
JW91	pJW81	*acfA3::lacZ*	VJ740	This work
JW93	pJW83	*acfD1::lacZ,ΔtoxT*	VJ740	This work
JW94	pJW84	*acfD2::lacZ,ΔtoxT*	VJ740	This work
JW96	pJW88	*acfD3::lacZ*	O395	This work
JW103	pJW95	*acfA14::lacZ*	O395	This work
JW104	pJW88	*acfD3::lacZ,ΔtoxT*	VJ740	This work
JW111	pJW95	*acfA14::lacZ,ΔtoxT*	VJ740	This work
JW112	pJW97	*acfD11::lacZ*	O395	This work
JW113	pJW98	*acfD12::lacZ*	O395	This work
JW118	pJW97	*acfD11::lacZ,ΔtoxT*	VJ740	This work
JW119	pJW98	*acfD12::lacZ,ΔtoxT*	VJ740	This work
JW136	pJW103	*acfA15::lacZ*	O395	This work
JW137	pJW104	*acfA16::lacZ*	O395	This work
JW138	pJW105	*acfD13::lacZ*	O395	This work
JW142	pJW103	*acfA15::lacZ,ΔtoxT*	VJ740	This work
JW143	pJW104	*acfA16::lacZ,ΔtoxT*	VJ740	This work
JW144	pJW105	*acfD13::lacZ,ΔtoxT*	VJ740	This work
JW153	pJW112	*acfA17::lacZ*	O395	This work
JW154	pJW113	*acfD14::lacZ*	O395	This work
JW158	pJW112	*acfA17::lacZ,ΔtoxT*	VJ740	This work
JW159	pJW113	*acfD14::lacZ,ΔtoxT*	VJ740	This work
JW205	pJW125	*acfD21::lacZ*	O395	This work
JW206	pJW126	*acfD22::lacZ*	O395	This work
JW220	pJW125	*acfD21::lacZ,ΔtoxT*	VJ740	This work
JW221	pJW126	*acfD22::lacZ,ΔtoxT*	VJ740	This work
JW242	pJW139	*acfD15::lacZ*	O395	This work
JW251	pJW139	*acfD15::lacZ,ΔtoxT*	VJ740	This work
JW252	pJW140	*acfA13::lacZ*	O395	This work
JW253	pJW140	*acfA13::lacZ,ΔtoxT*	VJ740	This work

mixed by shaking. Twenty millilitres of a 1:200 dilution of 3-Mercaptopropionic acid (Sigma) were then added to the gel tray and briefly mixed. The reaction continued for 7 min, and was stopped by addition of 20 ml 28 mM Neocuproine (Sigma), followed by shaking for 2 min. The gel was rinsed with 1000 ml of dH_2O and placed on X-ray film for 3 h. After the film was developed, bands corresponding to free DNA and H_6-ToxT/DNA complexes were excised from the gel based on their location in the film, and the gel slices were crushed and the DNA was eluted overnight in 0.5 ml of 0.5 M Ammonium Acetate, pH 7.5, 1 mM EDTA, 0.1% SDS, 10 mM $MgCl_2$. Gel pieces were pelleted by centrifugation, and the supernatant was passed through a 0.2 μm syringe filter and ethanol precipitated. The pellets were resuspended in a 1:1 mixture of TE:sequencing stop solution (USB) and radioactivity was measured with a Geiger counter. Approximately equal amounts of labelled DNA from the free DNA and H_6-ToxT/DNA complex bands were loaded on the subsequent sequencing gel. The sequencing ladder was produced with a Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (USB) as specified by the manufacturer, and the sequencing gel was prepared and run as specified by the sequencing kit manual. The same plasmid template and the primer that was end-labelled in the EMSA/footprinting experiment were used in the sequencing reactions. Autoradiography was performed with the resulting gel, and typical exposure times were 10–14 days.

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