

# Analysis of ToxR-dependent transcription activation of *ompU*, the gene encoding a major envelope protein in *Vibrio cholerae*

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## Summary

The membrane proteins ToxR and ToxS regulate a variety of genes associated with the virulence of *Vibrio cholerae*, the agent of human cholera. One of the ToxRS-regulated genes is the *ompU* gene, which encodes a porin that may also act as an adhesin. To begin to understand the mechanism of *ompU* transcription activation by ToxRS, we performed genetic and biochemical studies on the *ompU* promoter. Deletions with a 5' end-point at or downstream of –128, relative to the start site for transcription, did not direct expression of a *lacZ* reporter gene in wild-type *V. cholerae*, although the –128 promoter fragment did direct ToxRS-dependent reporter gene activity under conditions of ToxR overexpression in *E. coli*. Consistent with the activation data is that membranes containing ToxR and ToxS caused a gel electrophoretic mobility shift when mixed at low concentrations with deletion fragments whose end-point is at –211, but not with –128 or –68 fragments. ToxRS membranes did shift the –128 fragment when added at higher concentrations. DNase I footprinting analysis of *ompU* promoter DNA complexed with ToxRS membranes demonstrated protection of three sites: an upstream site ranging from –238 to –139, and two downstream sites ranging from –116 to –58 and –53 to –24. Within the DNA protected from DNase I digestion by ToxRS membranes, there are no elements bearing similarity to those identified previously within the promoters of two other ToxR-dependent genes, *ctxA* and *toxT*. We suggest a model for transcription activation that involves

sequential ToxR-binding events to distinct regions in the *ompU* promoter.

## Introduction

*Vibrio cholerae* causes the human diarrhoeal disease cholera. Pathogenic strains of *V. cholerae* express several virulence factors that are co-ordinately regulated by an inner membrane protein called ToxR. The genes encoding these factors are collectively termed the ToxR regulon, and include genes for the following: the cholera toxin (CTX), an ADP-ribosylating enzyme that intoxicates intestinal epithelial cells, resulting in the secretory diarrhoea associated with the disease (reviewed in Kaper *et al.*, 1995); the toxin co-regulated pilus (TCP), an adhesin that mediates intestinal colonization (Taylor *et al.*, 1987; Herrington *et al.*, 1988); the accessory colonization factor (ACF), which may play a role in signal transduction related to motility (Peterson and Mekalanos, 1988; Everiss *et al.*, 1994; Hughes *et al.*, 1994; 1995); and OmpU, a porin that may also function as an adhesin (Sperandio *et al.*, 1995; 1996; Chakrabarti *et al.*, 1996).

Although ToxR is localized in the membrane, it is a transcription factor with an amino-terminal cytoplasmic DNA binding/transcription activation domain and a carboxy-terminal periplasmic domain of undetermined function (Miller *et al.*, 1987; DiRita and Mekalanos, 1991; Ottemann *et al.*, 1992). The cytoplasmic domain of ToxR shares ≈30% homology at the amino acid level with the carboxy-terminal DNA-binding domain of OmpR and is therefore included in the OmpR family of transcription activators, which bind DNA by virtue of a winged helix–turn–helix domain (Ottemann *et al.*, 1992; Martínez-Hackert and Stock, 1997). Unlike OmpR, ToxR does not contain a phosphoacceptor domain and is believed to be activated in a manner other than phosphorylation. Activation may involve an additional membrane protein, ToxS, upon which ToxR is dependent for maximal activity (Miller *et al.*, 1989; DiRita and Mekalanos, 1991). ToxS has been hypothesized to stabilize a dimeric state of ToxR required for transcription activation (DiRita and Mekalanos, 1991; Dziejman and Mekalanos, 1994). Few regulatory proteins sharing the topological and functional features of ToxR have been described to date. *V. cholerae* has at least two proteins in this class, ToxR and the TcpP protein, a regulator

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encoded in the *tcp* gene cluster (Häse and Mekalanos, 1998). Other such regulators among different species of bacteria include CadC of *Escherichia coli* (Neely *et al.*, 1994), PsaE of *Yersinia pseudotuberculosis* (Yang and Isberg, 1997) and ToxR analogues of *Vibrio fischeri*, *Vibrio parahaemolyticus* and *Photobacterium* sp. strain SS9 (Lin *et al.*, 1993; Reich and Schoolnik, 1994; Welch and Bartlett, 1998). All of these proteins except CadC have a ToxS-like component associated with activity of the ToxR-like protein.

As mentioned above, expression of virulence factors is co-ordinately regulated by ToxR. Random insertions of the transposon *TnphoA* into the *V. cholerae* chromosome revealed that at least 17 genes are under ToxR regulation (Taylor *et al.*, 1987; Peterson and Mekalanos, 1988). However, several of these genes are not activated directly by ToxR, suggesting that another factor directly activates their transcription (DiRita *et al.*, 1991). This observation led to the discovery of ToxT, an AraC homologue that can activate transcription of several ToxR-regulated genes (DiRita *et al.*, 1991; Higgins *et al.*, 1992) and whose own transcription is regulated by ToxR, thus implying a cascade model of virulence gene regulation controlling virulence gene expression in *V. cholerae* (DiRita *et al.*, 1991; DiRita, 1992). ToxR controls *toxT* transcription by regulating a *toxT*-specific promoter (Higgins and DiRita, 1994), but *toxT* may also be autoregulated as the gene lies at the distal end of the operon encoding the ToxT-regulated *tcp* genes (Higgins and DiRita, 1994; Brown and Taylor, 1995). Recent work suggests that TcP and TcH, which are predicted to be membrane localized, are also involved in activation of *toxT* expression, perhaps in conjunction with ToxR or with another regulator (Carroll *et al.*, 1997; Häse and Mekalanos, 1998).

Several observations suggest that ToxR has other functions in virulence gene regulation besides activating *toxT* transcription. For example, expression of the *ctxAB* genes, which in *V. cholerae* are encoded on the genome of a filamentous converting phage called CTX $\Phi$  (Waldor and Mekalanos, 1996), can be activated in *E. coli* independently by ToxR or by ToxT, but *toxT* mutant strains of *V. cholerae* that express wild-type levels of ToxR are deficient for *ctxAB* transcription (Champion *et al.*, 1997). Thus, the exact role of ToxR in *ctxAB* expression remains uncertain, although a ToxR binding site within the *ctxAB* promoter has been identified and well characterized (Miller *et al.*, 1987; Pfau and Taylor, 1996).

Adding more complexity to virulence gene regulation by ToxR is the existence of a branch of the ToxR regulon comprising at least two genes that do not require ToxT for their expression (Champion *et al.*, 1997). These are *ompU*, which encodes the porin and putative adhesin OmpU mentioned above, and *ompT*, which, unlike the rest of the regulon, is under negative control by ToxR (Miller and Mekalanos, 1988).

The sequences of the two ToxR-regulated promoters that have been best described to date, those of the *ctxAB* and *toxT* genes, do not contain any conserved motifs suggestive of a consensus binding site that might be recognized by ToxR in activating their transcription. The distinguishing features of the *ctx* and *toxT* promoters, respectively, are a heptad, TTTTGAT, directly repeated three to eight times depending on the *V. cholerae* strain, and a set of three inverted repeats of sequence unrelated to the TTTTGAT motif (Miller *et al.*, 1987; Higgins and DiRita, 1994; 1996; Pfau and Taylor, 1996). The isolation of promoter point mutations, deletion analysis and DNase I footprinting demonstrated the presence of ToxR binding sites in these promoters, but failed to reveal the ToxR-binding code (Higgins and DiRita, 1996; Pfau and Taylor, 1996; J. A. Crawford and V. J. DiRita, unpublished).

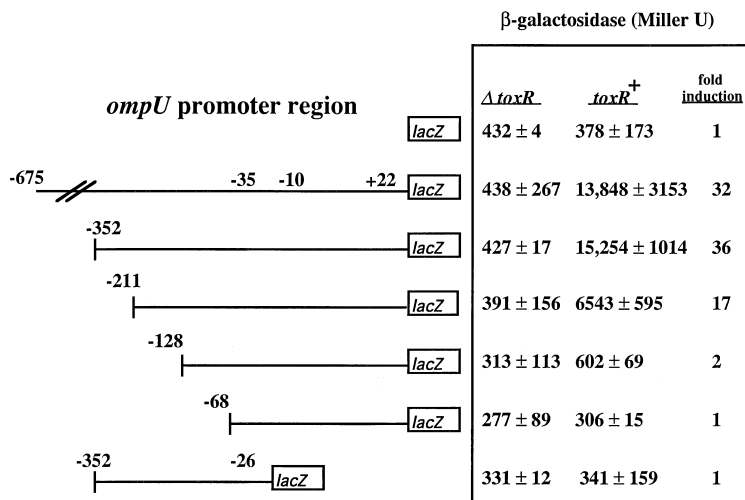
To gain further understanding of ToxT-independent regulation in *V. cholerae* and to continue clarifying the DNA-binding properties of ToxR, we investigated regulation of OmpU expression by ToxR. In this report, we present evidence that ToxR binds to the *ompU* promoter region and may directly activate *ompU* transcription. We also present data that identifies the ToxR-binding regions in this promoter and we propose a model for the molecular basis of ToxR-dependent transcription activation.

## Results

### Identification of a ToxR-regulated promoter upstream of *ompU*

Primer extension analysis of RNA isolated from *V. cholerae* wild-type classical strain O395 and its *toxR* mutant derivative (JJM43) revealed a ToxR-dependent transcription start site 159 nucleotides upstream of the *ompU* start codon (Sperandio *et al.*, 1996). To characterize the nature of this ToxR-dependent transcription, we constructed *ompU* reporter plasmids by fusing varying amounts of *ompU* upstream DNA to a promoterless *lacZ* gene in the plasmid TL61T. The 5' end-points for these fusions, relative to the ToxR-dependent transcription start site, are -675, -352, -211, -128, and -68 (each has a 3' end-point of +22). To remove the presumed -10 basal element, an additional construct was made in which the DNA extending from -353 to -26 was fused to *lacZ* (Fig. 1).

*ompU-lacZ* fusions were mobilized into classical strains O395 (wild type) and JJM43 (*toxR* mutant) *V. cholerae* and tested for their ability to direct synthesis of  $\beta$ -galactosidase in each background. As is seen in Fig. 1, constructs with upstream end-points at -675, -352 and -211 all directed high levels of ToxR-dependent  $\beta$ -galactosidase activity, whereas the -128 and -68 constructs expressed low levels of  $\beta$ -galactosidase in both wild-type and *toxR* mutant *V. cholerae*. As predicted, the -352 to



**Fig. 1.** Analysis of *ompU*–*lacZ* operon fusions in *V. cholerae*. Fusions were made by cloning PCR products containing portions of the *ompU* promoter in front of a promoterless *lacZ* gene on a broad host range plasmid. After determining that the PCR product sequence was wild type, the recombinant plasmids were introduced into *V. cholerae*. β-galactosidase activity was measured as described in the *Experimental procedures* and is reported in Miller Units (Miller, 1972).  $\Delta$ *toxR* and *toxR*<sup>+</sup> represent *V. cholerae* strains JMJ43 and O395 respectively. The 5' and 3' end-points of the *ompU* promoter region in each construct are labelled relative to the +1 transcription start site, as are the putative –35 and –10 RNA polymerase binding elements. Each strain was analysed at least three times.

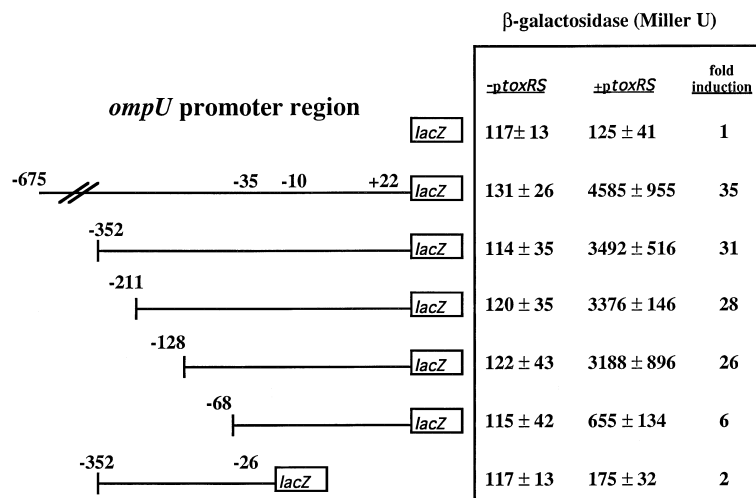
–26 construct, which deletes the putative basal promoter element at –10, expressed very low levels of β-galactosidase in both strain backgrounds. From these results, we conclude that the region upstream of –128 in the *ompU* promoter contains a ToxR-responsive regulatory element controlling transcription of the *ompU* gene that initiates at the position previously mapped (Sperandio *et al.*, 1996).

#### Transcription activation of *ompU* by *ToxRS* in *E. coli*

The dependence of *ompU* transcription on ToxR in *V. cholerae* suggests that either ToxR itself or a ToxR-regulated factor is the direct activator of *ompU* transcription. As described above, the vast majority of ToxR-regulated genes in *V. cholerae* are not activated directly by ToxR, but rather are activated by the ToxR-controlled ToxT activator. However, as *ompU* transcription is independent of ToxT (DiRita *et al.*, 1991; Champion *et al.*, 1997), we

developed the simple hypothesis that ToxR binds to and activates the *ompU* promoter directly without a requirement for other *V. cholerae* factors. To test this hypothesis, we assayed the β-galactosidase activity of the *ompU*–*lacZ* fusions in an *E. coli* background, reasoning that any factors that ToxR might require for function would probably not be present.

For this experiment, the *ompU* promoter deletion fragments described in Fig. 1 were subcloned in front of a promoterless *lacZ* in the plasmid RS551 and recombined onto the chromosome of *E. coli* strain TE2680 (Elliott, 1992). These various *ompU*–*lacZ* chromosomal fusion strains were transformed with pVJ21, a plasmid expressing ToxR and ToxS (Miller *et al.*, 1989), or with the parental plasmid ACYC184, and assayed for β-galactosidase activity. The results, shown in Fig. 2, demonstrate that ToxR and ToxS are sufficient for activation of *ompU* in *E. coli*, although they do not mirror what is seen in *V. cholerae* regarding the limits of ToxR dependence in the *ompU*

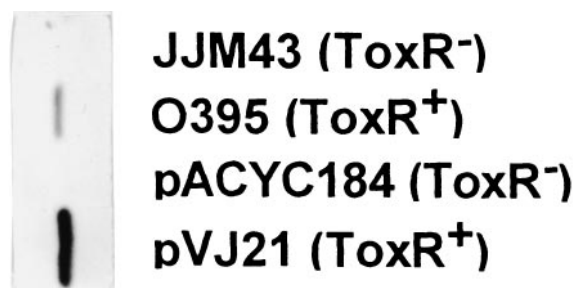


**Fig. 2.** Analysis of *ompU*–*lacZ* operon fusions in *E. coli*. *ompU* promoter fragments identical to those described in Fig. 1 were subcloned into pRS551 and recombined onto the chromosome of strain TE2680 as described in *Experimental procedures*. β-galactosidase activity was measured as described in *Experimental procedures* and is reported in Miller Units (Miller, 1972). –*ptoxRS* and +*ptoxRS* represent, respectively, chromosomal fusion strains containing pACYC184 or pVJ21. Each strain was analysed at least three times.

promoter. (This discrepancy will be addressed below.) Specifically, fusions with end-points, at/or, upstream of  $-128$  expressed high levels of  $\beta$ -galactosidase in the presence of pVJ21 but not in the presence of pACYC184. Compared with fusions containing greater amounts of upstream sequence, the  $-68$  to  $+22$  construct showed significantly lower levels of ToxR-dependent activation, but it was activated to an extent greater than that observed in *V. cholerae*. These results support our hypothesis that ToxR is the direct activator of *ompU* transcription, although they do not rule out the possibility that another factor, present in both *E. coli* and *V. cholerae*, may be involved.

The data from analysis of the promoter fusions in *V. cholerae* and *E. coli* present an apparent discrepancy because the  $-128$  fusion was activated to a high level in *E. coli* but was not expressed significantly above background in *V. cholerae*. A possible explanation for this is that although DNA upstream of  $-128$  may be required for the most efficient ToxR binding, overexpression of ToxR and ToxS from pVJ21 in *E. coli* might provide enough ToxR to recognize an otherwise poor binding site downstream of  $-128$ .

To determine the relative steady-state levels of ToxR in *E. coli* (pVJ21) and wild-type *V. cholerae*, we performed an immunoblot assay with anti-ToxR serum on total protein extracts normalized for culture growth. Bands on the immunoblot were quantified by densitometry, which showed that the steady-state level of ToxR was fourfold higher in *E. coli* (pVJ21) extracts than in *V. cholerae* wild-type extracts (Fig. 3). This confirms that ToxR is overexpressed in the *E. coli* background from pVJ21 and supports the hypothesis that the  $-128$  deletion may contain a weak ToxR



**Fig. 3.** Comparison of ToxR levels in *V. cholerae* and *E. coli*. *V. cholerae* strains are JJM43 (ToxR<sup>-</sup>) and O395 (ToxR<sup>+</sup>). The *E. coli* strain is TE2680 carrying the  $-675$  to  $+22$  chromosomal *ompU-lacZ* operon fusion and pACYC184 (ToxR<sup>-</sup>) or pVJ21 (ToxR<sup>+</sup>). Growth conditions were identical to those described in *Experimental procedures* for the  $\beta$ -galactosidase assays. Whole-cell lysates were prepared as described in *Experimental procedures* at the same OD<sub>600</sub> that the  $\beta$ -galactosidase assays were performed. OD<sub>600</sub> equivalents of total cellular protein were analysed by immunoblotting with ToxR polyclonal antisera as described in *Experimental procedures*. Each strain was analysed three times. The experiment was also performed using total cellular protein equivalents and the result was the same.

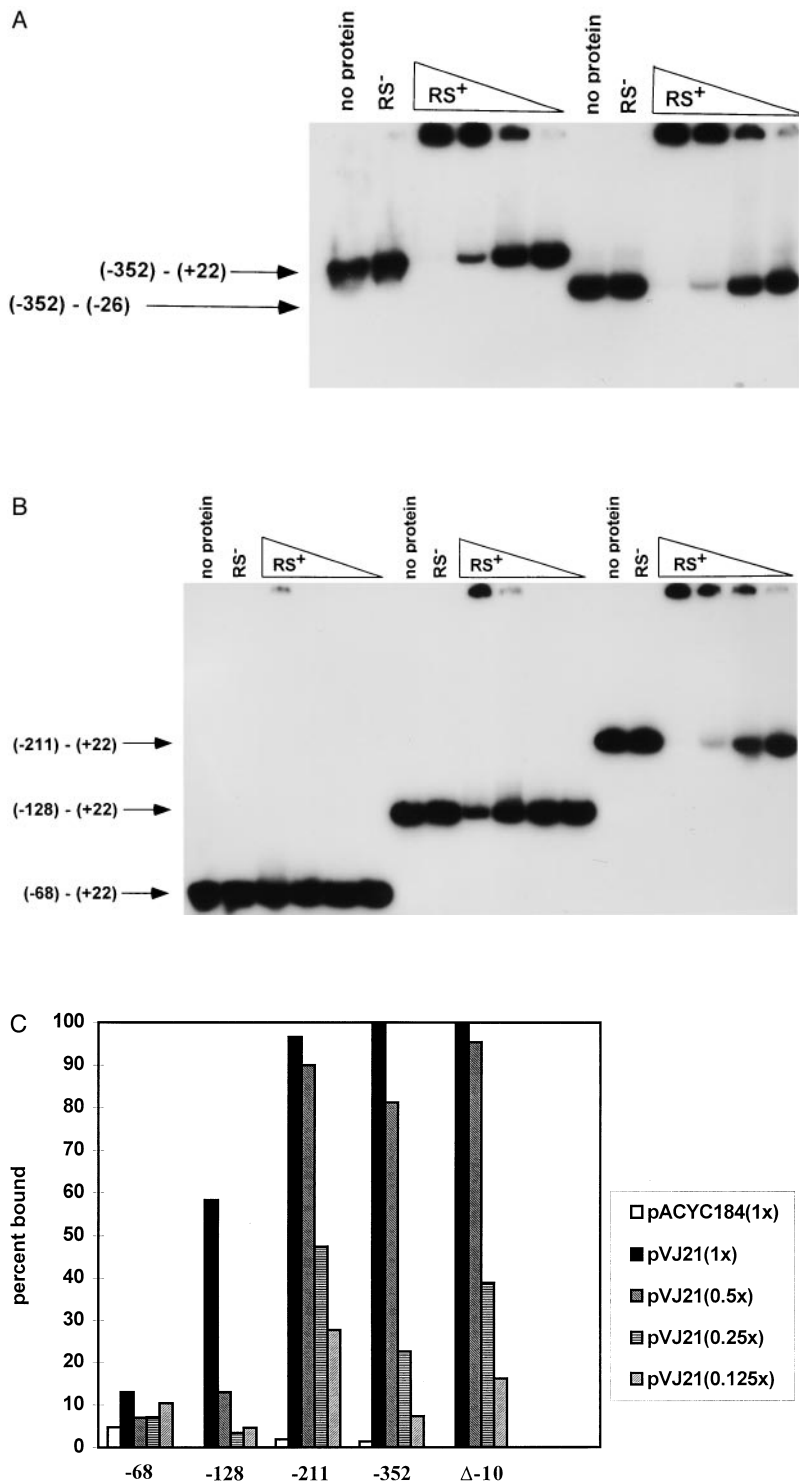
binding site that requires higher levels of the protein for activation. Further support for this hypothesis comes from the observation that the  $-128$  fusion is activated 18-fold in O395 harbouring pVJ21 (data not shown), and therefore overexpressing ToxR and ToxS, compared with only a twofold activation in the absence of ToxR overexpression (Fig. 1).

#### *Analysis of ToxR binding to the ompU promoter region*

Two other promoters in the ToxR regulon, the *toxT* and *ctxAB* promoters, harbour specific sequence elements to which ToxR binds. In the *ctxAB* promoter, the ToxR binding site includes a series of direct repeats with the sequence TTTTGAT (Miller *et al.*, 1987; Pfau and Taylor, 1996), whereas in the *toxT* promoter, ToxR recognizes DNA containing an inverted repeat sequence, rather than the direct repeats in the *ctxAB* promoter (Higgins and DiRita, 1994; 1996). To begin analysing the binding requirements for ToxR-dependent activation of the *ompU* promoter, we performed electrophoretic mobility shift assays using DNA fragments that correspond to specific regions upstream of *ompU*.

Deletion fragments used in the transcription analysis presented above were radiolabelled and incubated with membrane fractions of *E. coli* containing pVJ21 (ToxR<sup>+</sup>), or the parental plasmid ACYC184. The binding reactions were then subjected to electrophoresis on polyacrylamide gels as previously described (Higgins and DiRita, 1994). The binding reactions were performed with a series of membrane dilutions in order to obtain a semiquantitative estimate of how well they interact with specific promoter fragments. As can be seen in Fig 4A and B, although ToxRS membranes bind to DNA fragments having the  $-128$ ,  $-211$  and  $-352$  end-points, the most efficient binding, i.e. that which is observed at higher membrane dilutions, is to fragments with DNA upstream of  $-128$ . Thus, although ToxRS membranes bind with equivalent efficiency to the  $-352$  to  $+22$ ,  $-211$  to  $+22$  and  $-352$  to  $-26$  fragments, the  $-128$  to  $+22$  fragment was bound with reduced efficiency, as estimated by densitometric analysis of the gel shift (Fig. 4C). The negative control membranes lacking ToxR and ToxS do not bind to any of the probes, even when added at the highest protein concentration used for the ToxRS membranes. In addition, a non-specific DNA fragment competed at insignificant levels with the  $-352$  to  $+22$  probe, demonstrating that ToxRS membranes bind in a sequence-specific fashion to the *ompU* promoter (data not shown).

These results demonstrate that DNA upstream of  $-128$  is required for highest efficiency binding by ToxRS membranes and that DNA downstream of this position can be bound by ToxRS but with reduced efficiency in the absence of this upstream region. They are consistent



**Fig. 4.** Ability of *ToxR* to bind and shift *ompU* promoter DNA.

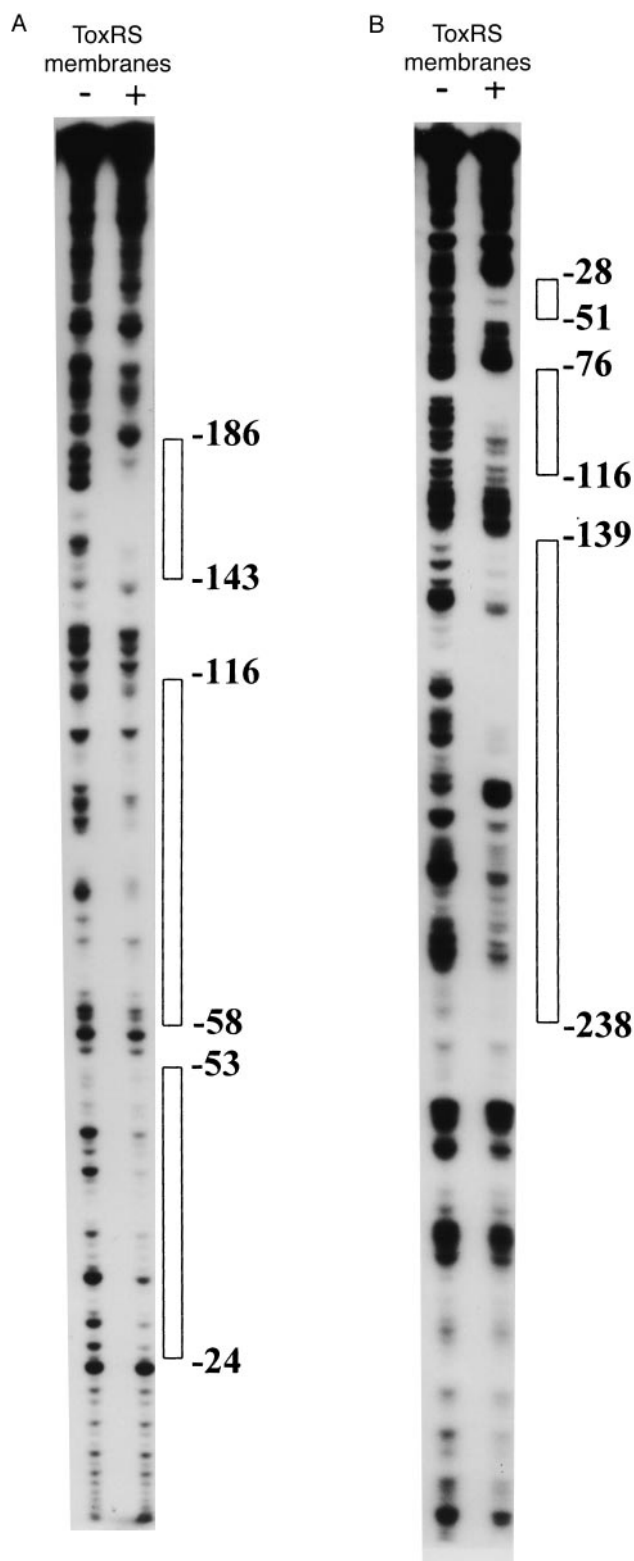
A. and B. Radiolabelled restriction fragments representing various regions of the *ompU* promoter were used in electrophoretic mobility shift assays as described in *Experimental procedures*. The numbers corresponding to the 5' and 3' end-points of each promoter fragment are relative to the +1 transcription start site. Labels at the top of the figure indicate whether DNA alone (no protein), membranes without *ToxR* and *ToxS* (RS<sup>-</sup>), or membranes containing *ToxR* and *ToxS* (RS<sup>+</sup>) were used in the assay. Decreasing protein concentration for the series of RS<sup>+</sup> reactions is indicated by the triangles.

C. Histogram of data from densitometric analysis of the shifted fragments shown in A and B. The percentage of the probe bound is measured on the y-axis. Probes are indicated on the x-axis. Δ-10 indicates the (-352) to (-26) probe. Relative protein concentrations are indicated in parentheses.

with the *ompU-lacZ* studies presented above in which the -128 fusion was activated in *E. coli* expressing *ToxR* from a multiple copy number plasmid, but not in *V. cholerae* expressing lower levels of *ToxR* from the chromosome.

*Determination of the ToxR binding sites in the ompU promoter by DNase I protection*

The results of the electrophoretic mobility shift assays predicted that the *ompU* promoter contains binding sites for



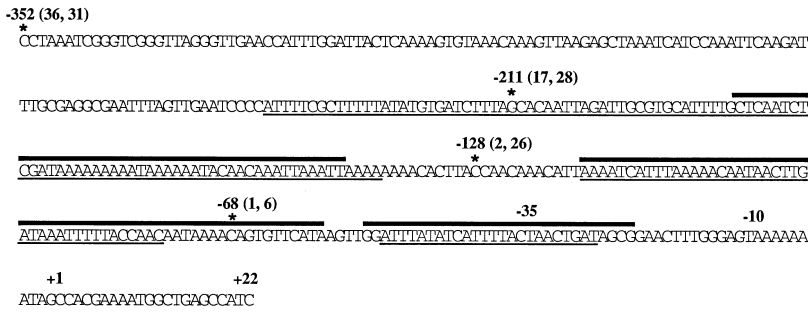
ToxR located both upstream and downstream of  $-128$ . To precisely map the position of the ToxR binding sites and to gain further insight into the sequence-specific requirements for ToxR binding to the *ompU* promoter, we performed DNase I footprinting experiments. The  $-352$  to  $+22$  fragment, which was bound with highest efficiency by ToxRS membranes and activated to maximal levels in both *V. cholerae* and *E. coli*, was radiolabelled and incubated with membrane fractions of *E. coli* containing pVJ21 (ToxRS<sup>+</sup>), or the parental plasmid ACYC184 as described in *Experimental procedures*. The binding reactions were next treated with DNase I, and subjected to electrophoresis on a denaturing polyacrylamide gel. As can be seen in Fig. 5A and B, three separate regions in the *ompU* promoter were protected from DNase I cleavage by ToxRS membranes; taking both strands into account, the limits of the protected regions are from  $-238$  to  $-139$ ,  $-116$  to  $-58$ , and  $-53$  to  $-24$ . The gels shown in Fig. 5A and B are representative of what we repeatedly observe in these footprinting experiments: that the two upstream sites are more strongly protected than the site at  $-53$  to  $-24$ . The DNase I cleavage pattern generated in the presence of negative control membranes (pACYC184) was identical to that of DNA alone (data not shown).

Consistent with the results of electrophoretic mobility shift assays (Fig. 4A and B) and the genetic analysis of the *ompU* promoter shown above, the footprint data show that ToxR binding sites are located both upstream and downstream of  $-128$  in the *ompU* promoter. A summary of the genetic and biochemical data presented above is shown in Fig. 6. Sequence analysis of the ToxR-binding regions in the *ompU* promoter did not reveal the presence of either the direct or inverted repeat sequences required for ToxR binding and transcription activation at the *ctxAB* and *toxT* promoters (Fig. 7). The possible implications for transcription activation resulting from ToxR binding within the *ompU* promoter are discussed below.

## Discussion

This report describes an analysis of the requirements for ToxR-dependent transcription of *ompU*, which encodes a major outer membrane protein and is in the ToxT-independent branch of the ToxR regulon. The simplest conclusion to be drawn from our results is that ToxR is the direct

**Fig. 5.** DNase I footprinting of ToxR on the *ompU* promoter. A. and B. Restriction fragments corresponding to the  $-352$  to  $+22$  promoter region, having been radiolabelled on one strand (A is the top strand, B is the bottom strand), were subjected to DNase I footprinting as described in *Experimental procedures*. Labels at the top of the figure indicate whether membranes without ToxR and ToxS ( $-$ ) or membranes containing ToxR and ToxS ( $+$ ) were used in the experiment. Bars to the side of the figure represent regions of the probe protected by ToxR and are labelled relative to the  $+1$  transcription start site.

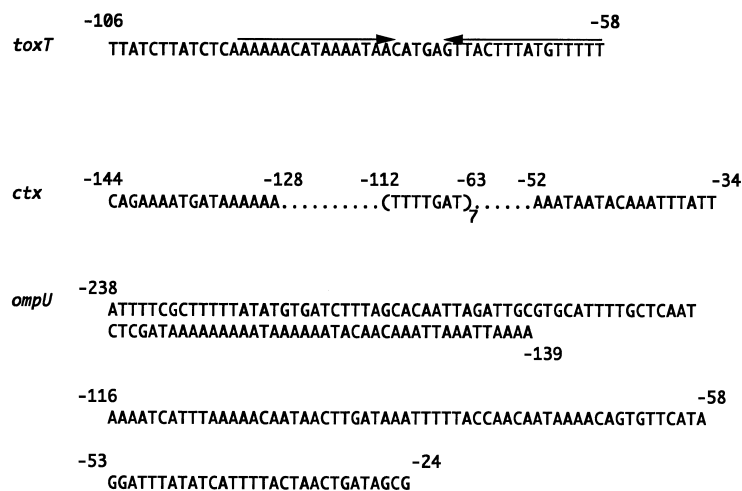


**Fig. 6.** Summary of the genetic and biochemical data regarding *ToxR* binding to and activation of the *ompU* promoter. The thick bars above and the thin lines below the sequence represent, respectively, the DNase I footprinting patterns generated on the top and bottom strands in the presence of *ToxR* (Fig. 5A and B). 5' deletion end-points are marked by an asterisk, whereas the numbers in parentheses represent the fold induction of transcription activation by *ToxR* in *V. cholerae* and *E. coli* (first and second numbers respectively) from Figs 1 and 2.

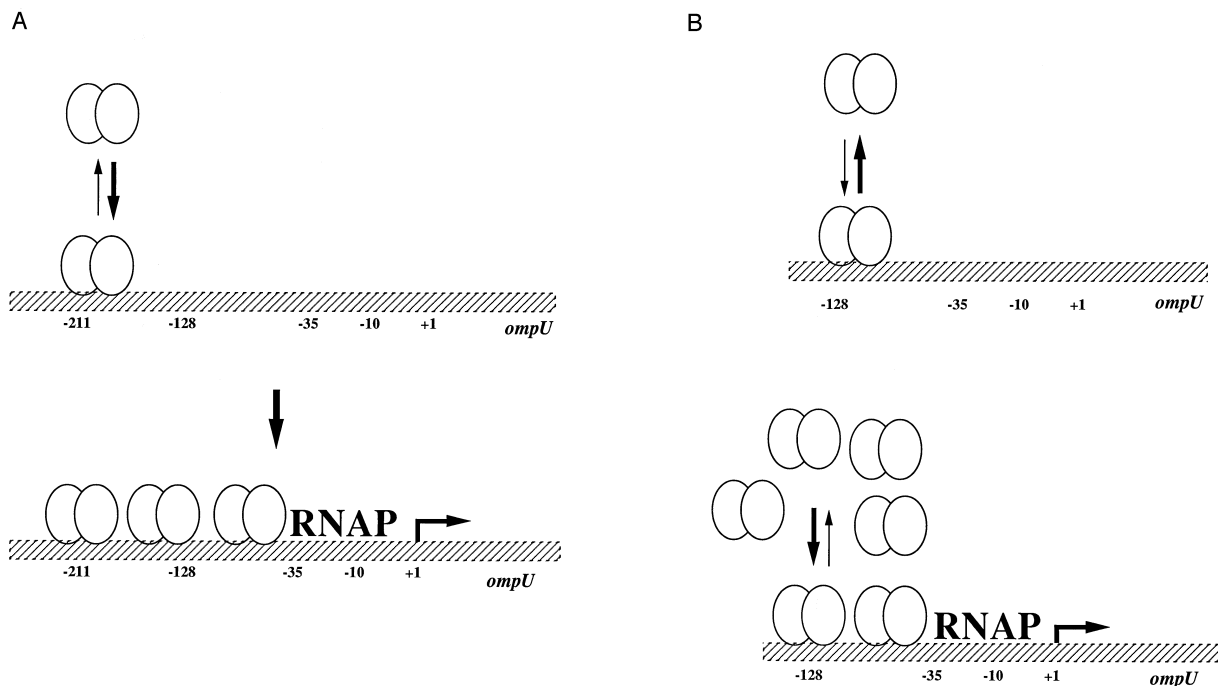
activator of *ompU*; this is based on the observations that *ToxR* directs high level expression of *ompU*–*lacZ* reporter fusions in *E. coli*, and that binding of *ToxR* to *ompU* promoter DNA correlates with its ability to activate expression in both *E. coli* and *V. cholerae*. Of the three genes whose promoters *ToxR* controls (*toxT*, *ctxAB* and *ompU*), the data for *ompU* are the most straightforward to interpret when trying to ascertain the molecular mechanism for transcription activation by *ToxR*. Data regarding the expression of *ompU* in *E. coli* and *V. cholerae* are generally quite consistent between the two species. In contrast, disparities between what is observed in *V. cholerae* and in *E. coli* make it difficult to arrive at a simple conclusion regarding how the *ctxAB* and *toxT* promoters may be regulated by *ToxR*. For example, the *ctxAB* promoter is activated to high levels in *E. coli* with either *ToxR* or *ToxT* supplied *in trans* (Miller and Mekalanos, 1984; DiRita *et al.*, 1991) but is not detectably expressed in *V. cholerae* in the absence of functional *ToxT*, irrespective of the presence of *ToxR* (Champion *et al.*, 1997). Also, although *toxT* expression requires *ToxR* in *V. cholerae*, a *toxT*–*lacZ* gene fusion was not activated in *E. coli* by *ToxR*, suggesting that another *Vibrio* factor may be required for *toxT* expression (Higgins and DiRita, 1994). Indeed, recent work has shown that two other gene products in the *tcp*

gene cluster, *TcpP* and *TcpH*, play a role in *ToxR*-dependent *toxT* expression (Carroll *et al.*, 1997; Häse and Mekalanos, 1998). That *ompU* activation may be the most direct function for *ToxR* is consistent with a model (Champion *et al.*, 1997) in which *ToxR* evolved to control *ompU* and *ompT* expression, and that its more indirect regulation of *ctxAB* and *tcp* arose upon acquisition of those gene clusters at a later time (Kovach *et al.*, 1996; Waldor and Mekalanos, 1996).

Figure 8 presents a model for how *ToxR* interacts with the *ompU* promoter to activate its transcription. This model is derived in part from a hypothesis proposed by Pratt and Silhavy (1996) for how *OmpR* activates transcription and is also similar to a model proposed by Stibitz and colleagues for transcription activation by *BvgA*, a response regulator that controls the virulence of *Bordetella pertussis* (Boucher and Stibitz, 1995; Boucher *et al.*, 1997). We propose that *ToxR* binding to sites in the –238 to –139 region promotes binding to sites in the –116 to –58 and –53 to –24 regions that results in transcription activation, presumably through interaction between *ToxR* and RNA polymerase (Fig. 8A). The model is based on our observation that deletion of sequences upstream of –128 result in a weaker promoter that requires overexpression of *ToxR* to be activated (Fig. 8B). The precise mechanism



**Fig. 7.** Sequences of *ToxR* binding sites in the *toxT*, *ctx* and *ompU* promoters. The *ToxR* binding site in the *toxT* promoter consists of an inverted repeat plus a portion of the downstream half of an adjacent inverted repeat (Higgins and DiRita, 1994; 1996; J. A. Crawford and V. J. DiRita, unpublished), whereas in the *ctx* promoter *ToxR* binds to a direct repeat plus flanking sites (Miller *et al.*, 1987; Pfau and Taylor, 1996; J. A. Crawford and V. J. DiRita, unpublished).



**Fig. 8.** Model for ToxR binding and activation of the *ompU* promoter. The pairs of ovoid symbols represent ToxR. RNAP indicates RNA polymerase.  
 A. Binding and activation of the full-length *ompU* promoter requires sequences upstream of  $-128$  for wild-type levels of ToxR.  
 B. Binding to the mutant promoter lacking sequences upstream of  $-128$  results in activation only under conditions of ToxR overexpression. See text for details.

for the role of upstream binding by ToxR is not clear from the current data, but we consider it unlikely that upstream binding is required to place ToxR in phase on the helix with RNA polymerase. If that were the case the promoter with a  $-128$  upstream end-point would not be expected to activate transcription whether or not ToxR were overexpressed. We hypothesize that ToxR bound at the upstream site recruits other ToxR proteins to the downstream sites through protein-protein interaction, which may involve co-operative binding by ToxR. This type of mechanism might provide a rationale for the predicted multimeric structure of functional ToxR (Miller *et al.*, 1987; DiRita and Mekalanos, 1991; Dziejman and Mekalanos, 1994). The model shown in Fig. 8 makes predictions about the affinity of ToxR for each individual binding region and the contributions of each binding region to transcription activation by ToxR, and these are currently being tested.

Our data demonstrate the presence of ToxR binding sites required for transcription activation of the *ompU* promoter, but the sequence of the ToxR-binding regions in the *ompU* promoter does not include elements found in the *ctx* and *toxT* promoters (Fig. 7). The model for evolution of this system alluded to above suggests that co-ordinate control of these three promoters by ToxR is a result of convergent evolution (Champion *et al.*, 1997), which might account for this lack of obvious sequence similarity.

Evolutionary considerations aside, it is instructive to explore possible mechanisms by which ToxR may recognize these three different promoters as an apparent requirement for their activation. There may be a conserved sequence required for ToxR binding in all three promoters that could be difficult to identify simply by analysing the primary sequences, perhaps in part because of the overall A+T richness of the ToxR binding sites in all three promoters. A consensus site masked in this way would be particularly difficult to identify if it were degenerate. This seems to be the case for OmpR binding sites, which share little sequence conservation (Huang and Igo, 1996; Pratt and Silhavy, 1996). Second, ToxR may recognize a structural feature of the DNA, rather than (or in addition to) a specific primary sequence. Two facts about the *ompU* promoter lead us to consider this as a distinct possibility. This promoter is highly A+T rich, with several runs of A residues, and such DNA is predicted to be bent (Haran *et al.*, 1994). Also, DNase I digests the ToxR binding site with reduced efficiency (Fig. 5; negative control reactions), suggesting that DNA in this region is less accessible to the enzyme. This is perhaps due to distortions in the minor groove where DNase I recognizes and binds DNA (Suck, 1994). That ToxR binds and protects these regions is consistent with the hypothesis that it may recognize distorted DNA.



## Experimental procedures

### Bacterial strains and plasmids

The *V. cholerae* and *E. coli* strains and plasmids used in this study are listed in Table 1. Strains were grown in Luria broth (LB) medium at 30°C. The strains were maintained at -70°C in LB medium plus 20% glycerol. Antibiotics were used at the following concentrations: ampicillin, 100 mg ml<sup>-1</sup>; tetracycline, 12.5 mg ml<sup>-1</sup>; kanamycin, 30 mg ml<sup>-1</sup>; chloramphenicol, 50 mg ml<sup>-1</sup>; and streptomycin, 100 mg ml<sup>-1</sup>. Plasmids were introduced into *E. coli* strains by transformation and into *V. cholerae* strains through triparental mating with *E. coli* strain MM294(pRK2013) as a donor of mobilization functions.

### DNA manipulations

Plasmid DNA was purified with Qiagen columns (Qiagen). PCR was performed using the Expand High Fidelity PCR System (Boehringer Mannheim) as specified by the manufacturer. PCR templates were either a λ-ZAP clone of *ompU* DNA (Sperandio *et al.*, 1996) or plasmid AC113, containing the *ompU* promoter region extending from -675 to +232. When necessary, synthesized primers contained added recognition sequences for restriction endonucleases to facilitate directional cloning. PCR products were purified by agarose gel electrophoresis followed by gel extraction with the QIAEX II gel extraction system (Qiagen). Cloning was performed using standard protocols (Sambrook *et al.*, 1989). Double-stranded sequencing of plasmid DNA was sequenced as previously described (Higgins and DiRita, 1994).

### Transcriptional fusion analysis

PCR products harbouring various portions of the *ompU* promoter region were generated with flanking *EcoRI* and *BamHI* sites and subcloned into *EcoRI*-*BamHI*-digested pTL61T. After verifying the sequences to be wild type, plasmid DNA was transferred into *V. cholerae* strains O395 and JJM43.

*V. cholerae* cells containing plasmids were grown overnight in LB medium with a starting pH of 8.5 at 30°C then diluted 1:50 in fresh LB medium with a starting pH of 6.5 and grown at 30°C for 2.5 h. Cells were harvested and β-galactosidase activity was measured as described (Miller, 1972).

### Construction of *ompU*-*lacZ* chromosomal fusions in *E. coli*

The *ompU*-*lacZ* chromosomal fusion strains were constructed using the method of Elliot (1992). Briefly, the same PCR fragments cloned into pTL61T were subcloned into *EcoRI*-*BamHI* digested pRS551 and linearized by digestion with *XhoI*. Linear DNA was transformed into *E. coli* strain TE2680 and cells were selected for kanamycin resistance (Kan<sup>R</sup>) and chloramphenicol sensitivity (Cm<sup>S</sup>), which indicates recombination of the fusion onto the chromosome. pVJ21 (ToxRS<sup>+</sup>) and the parental vector pACYC184 were separately transformed into each *ompU*-*lacZ* chromosomal fusion strain and β-galactosidase activity was measured as for *V. cholerae* strains with the following exception: the starting pH of the media was always 6.5 as there is no observable effect of pH on *ToxR*-regulated gene expression in *E. coli*.

### *ToxR* immunoblotting

Whole-cell lysates of each strain were prepared by growing each strain as described above in the β-galactosidase assay protocol. Each overnight culture was back-diluted 1:50 and grown for 2.5 h at 30°C. A 1 ml sample of each culture was pelleted, resuspended in SDS-PAGE sample buffer and boiled for 5 min. OD<sub>600</sub> equivalents of total cellular protein were electrophoresed on a 10% polyacrylamide gel with a 5% stacking gel, transferred to nitrocellulose and probed with *ToxR* polyclonal antisera as previously described (DiRita *et al.*, 1996). Parallel gels loaded with the same sample volumes were electrophoresed and stained with Coomassie brilliant blue to ensure equal loading on the blotted gel.

**Table 1.** Strains and plasmids used in this study.

Strain/plasmid	Description	Reference/source
<i>Strain</i>		
<i>V. cholerae</i>		
O395	Str <sup>R</sup>	Laboratory collection
JJM43	O395 Δ <i>ctxA1</i> Δ <i>toxR43</i>	Herrington <i>et al.</i> (1988)
<i>E. coli</i>		
TE2680	F <sup>-</sup> λ <sup>-</sup> IN( <i>rrnD</i> - <i>rrnE</i> ) 1 Δ( <i>lac</i> )X74 <i>rpsL</i> <i>galK2recD1903</i> ::Tn10d-Tet <i>trpDC700</i> :: <i>putPA1303</i> ::(Kan <sup>S</sup> -Cam <sup>R</sup> - <i>lac</i> )	Elliott (1992)
AAEC189	F <sup>-</sup> Δ <i>fim</i> <i>recA</i> <i>supE44</i> <i>hsdR17</i> <i>mcrA</i> <i>tmcrB</i> <i>endA1hi-1</i> Δ( <i>argF</i> - <i>lac</i> )205(U169) λ <sup>-</sup>	Blomfield <i>et al.</i> (1991)
<i>Plasmid</i>		
pTL61T	<i>lacZ</i> transcriptional fusion vector; Ap <sup>R</sup>	Linn and Pierre (1990)
pACYC184	Cloning vector; Tc <sup>R</sup> Cam <sup>R</sup>	Chang and Cohen (1978)
pVJ21	pACYC184 <i>tc::toxRS</i> ; Cam <sup>R</sup>	Miller <i>et al.</i> (1989)
pRS551	<i>lacZ</i> transcriptional fusion vector; Ap <sup>R</sup> Kan <sup>R</sup>	Simons <i>et al.</i> (1987)

Str<sup>R</sup>, streptomycin resistant; Ap<sup>R</sup>, ampicillin resistant; Tc<sup>R</sup>, tetracycline resistant; Kan<sup>R</sup>, kanamycin resistant; Cam<sup>R</sup>, chloramphenicol resistant.

### Preparation of membrane fractions

*E. coli* strain AAEC189 containing either pVJ21(ToxRS<sup>+</sup>) or pACYC184 were grown overnight at 30°C in LB media. Cells were collected by centrifugation, resuspended in lysis buffer (20 mM KH<sub>2</sub>PO<sub>4</sub>, 50 mM KCl, 5 mM EDTA, pH 7.8, 1 mM leupeptin, 1 mM pepstatin A, 16.7 mM phenylmethylsulphonyl fluoride) and passed through a French Press twice. Unbroken cells were removed by centrifugation at 350 × *g* for 15 min. The lysate was loaded onto a 15%/70% discontinuous sucrose gradient and centrifuged at 36 000 r.p.m. in a SW41 swinging bucket rotor for 90 min. The membrane fraction was removed from the 15%/70% interface and resuspended in cold 20 mM KH<sub>2</sub>HPO<sub>4</sub>, 1 mM EDTA, pH 7.8. Membranes were pelleted by centrifugation at 36 000 r.p.m. in a SW41 swinging bucket rotor for 90 min and then resuspended in cold 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM EDTA, pH 7.8. Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad).

### DNA gel mobility assays

*ompU* promoter fragments, having been subcloned into pBlue-script SK+/- (Stratagene), were isolated as DNA probes for the experiments described in Fig. 4A and B by first linearizing plasmid DNA with *Sa*I and subjecting these samples to an end-labelling reaction containing dATP, dGTP, dTTP, each at 2 mM, 30 μCi of [ $\alpha$ -<sup>32</sup>P]-dCTP (>3000 Ci mmol<sup>-1</sup>, Amersham), and 5 units of Klenow (New England Biolabs). Reactions were incubated at room temperature for 5 min, followed by 70°C for 15 min. Samples were next digested with *Sa*cl, which cuts the probe fragment out of the vector. Next, reactions were electrophoresed on a 1 × TBE, 5% polyacrylamide gel and subjected to autoradiography. Probe fragments were excised out of the gel and eluted in 0.5 M ammonium acetate, pH 7.5, 0.1% SDS, 1 mM EDTA, pH 8.0, overnight at 37°C. Next, the samples were ethanol precipitated and then used in gel shift reactions. Retardation of radioactively labelled DNA fragments was performed as previously described (Miller *et al.*, 1987) with the following modifications. Assays were performed in 20 μl volumes with membrane fractions containing 500 mg ml<sup>-1</sup> protein for the negative control reaction (ToxRS<sup>-</sup> membranes), and 500 μg ml<sup>-1</sup>, 250 μg ml<sup>-1</sup>, 125 μg ml<sup>-1</sup> and 62.5 μg ml<sup>-1</sup> protein for the experimental reactions (ToxRS<sup>+</sup> membranes). Each reaction contained 10 μg ml<sup>-1</sup> salmon sperm DNA and 3000 c.p.m. of probe. The binding reactions were incubated at 30°C for 30 min. Autoradiographs were quantified directly using an AMBIS 4000 scanner set for densitometric reading.

### DNase I footprinting

Footprinting probes were generated by cutting pBluescript SK+/- (Stratagene) containing the -352 to +22 *ompU* promoter region with either *Sa*ll (for labelling the bottom strand) or *Not*I (for labelling the top strand), subjecting these samples to the end-labelling reaction described above in the DNA gel mobility assay, and cutting the probe fragment out of the vector with either *Sa*cl (bottom strand probe) or *Sa*ll (top strand probe). Probes were isolated and purified as described above in the DNA gel mobility assay. Binding reactions were

performed as described above in the DNA gel mobility assay with the following modifications. Reactions were set up in 70 μl volumes containing 1000 μg ml<sup>-1</sup> protein for the negative control (ToxRS<sup>-</sup> membranes) and experimental reactions (ToxRS<sup>+</sup> membranes). Each reaction contained 70 000 c.p.m. of probe. Under these conditions, 100% of the probe is bound by ToxR, as measured by electrophoretic mobility shift assays. To the binding reactions, CaCl<sub>2</sub> and MgCl<sub>2</sub> were added to final concentrations of 1 mM and 5 mM respectively. DNase I (0.01 units) (Boehringer Mannheim) was next added and incubated at room temperature for 2 min. The reactions were stopped by addition of a stop solution consisting of 200 mM NaCl, 2 mM EDTA and 1% SDS, followed by three phenol-chloroform extractions. Reactions were next ethanol precipitated, washed twice with 70% ethanol and electrophoresed on a 5% polyacrylamide, 6.9 M urea gel. To precisely map the position of the ToxR-binding regions in the *ompU* promoter, a sequencing reaction was performed (Amersham Life Sciences, T7 Sequenase Version 2.0 DNA sequencing kit) on the appropriate strand of the -352 to +22 *ompU* promoter clone in which the 5' end of the sequencing primer corresponds to the 5' end of the footprinting probe. The sequencing reaction was electrophoresed alongside the footprinting reactions.

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