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Identification of *toxS*, a Regulatory Gene Whose Product Enhances ToxR-Mediated Activation of the Cholera Toxin Promoter

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We describe the cloning of the *toxS* gene from *Vibrio cholerae* E1 Tor strain E7946. This gene lies downstream from the *toxR* gene, which encodes the transcriptional activator for the cholera toxin (*ctx*) operon in *V. cholerae*. We show that ToxS acts in conjunction with ToxR to activate expression of the *ctx* operon in *Escherichia coli*. The classical strain 569B, which is attenuated for virulance but which synthesizes high levels of cholera toxin in vitro, carries a deletion of 1.2 kilobase pairs of DNA, downstream from the *toxR* gene, which removes *toxS*. We present evidence that *toxS* is the downstream gene in an operon with *toxR*.

Many strains of *Vibrio cholerae* synthesize a heat-labile protein known as cholera toxin, which induces diarrhea in infected individuals and is primarily responsible for the disease called Asiatic cholera (4). Cholera toxin is a multimeric protein composed of an A subunit (28 kilodaltons) and five identical B subunits (11.5 kilodaltons) which are encoded by the *ctxAB* operon (6, 18). This operon is part of a larger genetic element which is often found duplicated or amplified in *V. cholerae* (9).

We previously described a gene, *toxR*, whose product is responsible for activation of the *ctxAB* operon (13). ToxR is a 38-kilodalton, transmembrane, DNA-binding protein capable of binding to a repeated sequence, TTTTGAT, located 77 base pairs (bp) upstream of the start of *ctxAB* transcription (16). Genetic and structural analyses have shown that the previously identified *tox* regulatory locus is *toxR* (5, 7, 14).

In addition to its role in activation of *ctxAB* expression, ToxR is required for the activation of several other virulence-related genes in *V. cholerae*. These include the *tcpA* gene, whose product is the major subunit of the toxin coregulated pilus colonization factor TCP; several genes involved in pilus assembly and export; and genes of the *acf* locus, which is responsible for elaboration of an accessory colonization factor (19, 26). Furthermore, osmoregulation of two outer membrane proteins, OmpT and OmpU, is mediated by ToxR (15). The response of *V. cholerae* to in vitro environmental conditions (NaCl concentration, the presence of certain amino acids, pH, and temperature) which are known to affect the expression of the gene products described above requires ToxR (3, 15, 16, 20), suggesting that this protein is the major component of signal transduction important for the coordinate expression of virulence genes in *V. cholerae*.

The *toxR* gene was originally cloned from the classical Inaba strain 569B, a strain which produces more toxin in vitro yet is generally less virulent than other strains of *V. cholerae* (13). When a Southern blot of DNA from several strains of *V. cholerae* was probed with the cloned *toxR* gene from 569B, *toxR* homologous sequences were detected in all cases, although the hybridizing fragment was 1.2 kbp larger in the more virulent strains than it was in strain 569B (14). Genetic experiments suggested that the 1.2-kbp deletion in 569B is associated with increased toxin production by this strain (14). To examine the differences between the *toxR* locus from 569B and from other strains of *V. cholerae*, we cloned the *toxR* locus from the El Tor strain E7946, a strain which produces low amounts of toxin in vitro. In this report, we present evidence that the 1.2-kbp deletion in 569B removes a gene, *toxS*, whose product acts in *trans* and is required for activation of expression of the *ctxAB* gene by ToxR.

MATERIALS AND METHODS

Bacterial strains and media. *V. cholerae* and *E. coli* strains were maintained at −70°C in LB containing 25% (vol/vol) glycerol or on LB plates (12). The strains and their genotypes are listed in Table 1. Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; tetracycline, 15 μg/ml; chloramphenicol, 15 μg/ml.

Nucleic acid preparation and analysis. Northern (RNA) blot analysis was performed as previously described (13). The blot used in this study had been used previously for analysis of *ctx* transcripts (13); probe was removed by rehydrating in 1× SSC (0.15 M NaCl plus 0.015 sodium citrate), rinsing in 0.1× SSPE–0.1% sodium dodecyl sulfate (at 65°C) (20× SSPE is composed of 174 g of NaCl, 27.6 g of $NH_2PO_4 \cdot H_2O$, and 7.4 g of EDTA per liter), and then briefly rinsing in 1× SSC (27).

DNA restriction enzymes and phage T4 DNA ligase were purchased from New England BioLabs, Inc. (Beverly, Mass.). Calf intestinal alkaline phosphatase was purchased from Boehringer-Mannheim (Indianapolis, Ind.). Conditions used were those suggested by the manufacturer.

β-Galactosidase assays. β-Galactosidase activity was assayed as detailed by Miller (12) and as described in the footnotes to Table 2 in this report.

RESULTS

Cloning of the *toxR* gene from strain E7946. Plasmid DNA from a library of *V. cholerae* E7946 DNA, partially digested with *Sau*3A and inserted into *Bam*HI-digested pBR327, was used to transform the Δ*lac E. coli* strain VM2, which carries a chromosomal *ctx*::*lacZ* fusion; all β-galactosidase activity produced by VM2 is the result of expression of *lacZ* from the *ctx* promoter (13). The transformed cells were spread on

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TABLE 1. Strains, plasmids, and phage used in this study

Strain, plasmid, or phage	Genotype	Reference(s)
E. coli		
SY327	F⁻ *araD* Δ(*lac pro*) *argE*(Am) Rif^r *nalA recA56*	(8)
VM2	SY327 (λ NFVM1)	(13)
V. cholerae		
569B	Prototrophic	Laboratory collection
E7946	Prototrophic	Laboratory collection
O395	Prototrophic	Laboratory collection
M7922	Δ*ctxAB*	(10)
M13	*toxR*	(5, 14)
Phage		
λNFVM1	*c*I857 *S*am100 *int*$^+$ *att*$^+$ *ctx-lacZ*	(13)
Plasmids		
pJM290.2	*ctxA23* Tc^r	(11)
pACYC184	Tc^r Cm^r	(1)

lactose-MacConkey plates containing ampicillin. VM2 is normally light pink on lactose-MacConkey plates, but activation of the *ctx*::*lacZ* gene fusion results in a dark pink colony color. Six colonies exhibiting the activation phenotype were identified and assayed for β-galactosidase activity. Restriction endonuclease analysis of plasmid DNA from these strains revealed that all six carried the *toxR* gene, including the 756-bp *Eco*RI-*Nco*I fragment known to represent most of the coding sequence of the 569B *toxR* gene (16), as well as the 1.2 kbp of downstream DNA. Two *toxR*-containing recombinant plasmids, pVM48 and pVM49, were characterized further. Strains harboring these plasmids showed three- to fivefold activation of β-galactosidase expression from the *ctx* promoter. In addition to activating the *ctx*::*lacZ* gene fusion, pVM48 and pVM49 could activate expression of *ctxB* from the *ctxA* promoter in pJM290.2 (data not shown; 13). To confirm that the *ctx*::*lacZ* activation phenotype was plasmid associated and to demonstrate that we had not cloned a *V. cholerae* β-galactosidase, pVM48 and pVM49 were used to transform VM2 and SY327, a strain of *E. coli* which is deleted for *lacZ* (8). No β-galactosidase activity was detected in SY327 transformants, whereas a 3.5-fold induction of β-galactosidase was again observed in VM2 transformants (Table 2). Maxicell and DNA sequence analyses confirmed that pVM48 and pVM49 harbor the *toxR* gene (data not shown).

Construction and analysis of 569B-E7946 hybrid *toxR* plasmids. The *toxR* loci from E7946 and 569B are structurally different due to the 1.2 kbp of DNA present in E7946 which is absent in 569B. The observation that *toxR*-containing plasmids derived from 569B activate *ctx*::*lacZ* expression 15-fold (13) while the E7946 *toxR*-containing plasmids activate *ctx*::*lacZ* only 3- to 5-fold suggested that there also may be a functional difference between *toxR* loci from these two strains. To define the differences between the *toxR* loci from 569B and E7946, we made hybrid plasmids of four types: (i) a plasmid that has the 569B *toxR* structural gene replaced with the E7946 *toxR* structural gene (pVM32); (ii) a plasmid with the 569B *toxR* gene and the E7946 sequences downstream from *toxR* (pVM33); (iii) a plasmid with the 569B *toxR* gene and E7946 sequences upstream from *toxR* (pVM34); and (iv) a plasmid with the 569B *toxR* gene and E7946 sequences both upstream and downstream from *toxR* (pVM35; Fig. 1).

TABLE 2. Activation of the *ctx-lacZ* operon fusion by E7946 *toxR* clones[a]

Strain	β-Galactosidase activity[b] (U)	Activation[c]
VM2	43	1.0
VM2(pVM48)	149	3.5
VM2(pVM49)	152	3.5

[a] *E. coli* cells grown to late exponential phase in LB with shaking at 30°C were used for β-galactosidase assays (12).
[b] Units are as described (12).
[c] Activation is calculated as activity/activity of VM2.

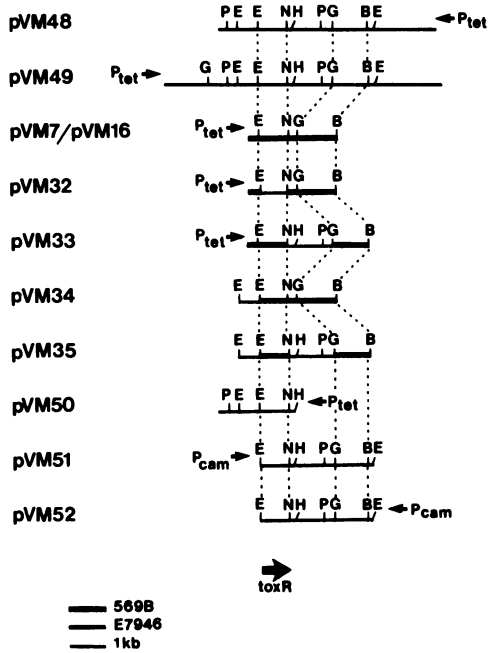

FIG. 1. Restriction enzyme maps of recombinant plasmids carrying the *toxR* gene. The heavy arrow below the restriction maps shows the region of DNA that encodes the *toxR* gene. The direction of the arrow indicates the direction of transcription of *toxR*. The *toxR* gene begins 158 bp upstream of the *Eco*RI site and ends 35 bp upstream of the *Nco*I site. The location and direction of transcription of promoters located in the vectors are also shown. The following letters are used to represent different restriction enzyme sites on the maps: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; N, *Nco*I; P, *Pst*I.

Plasmid pVM32 was constructed to assess any differences between the 569B and E7946 *toxR* structural genes. In plasmid pVM32, the 756-bp *Eco*RI-*Nco*I fragment of pVM16 (a pVM7 [13] derivative with the pBR322 *Eco*RI site filled in) is replaced with the 756-bp fragment purified from pVM49 (Fig. 1). This fragment represents ToxR coding information from amino acid 53 through the end of the protein; ToxR synthesized from pVM32 is thus composed largely of E7946 *toxR* coding information (16; Fig. 1). pVM7 and pVM32 both activated *ctx* to a high level (Table 3), indicating that the E7946 *toxR* polypeptide sequences in the hybrid work at least as well as the corresponding 569B sequences.

In pVM33, the 245-bp *Nco*I-*Bgl*II fragment containing DNA downstream of the *toxR* gene in pVM7 was replaced with the 1.4-kbp *Nco*I-*Bgl*II fragment from pVM49 (Fig. 1).

TABLE 3. Activation of the *ctx-lacZ* operon fusion by 569B-E7946 hybrid *toxR* genes

Strain	β-Galactosidase activity (U)	Activation
VM2	70	1.0
VM2(pVM7)	1,031	14.7
VM2(pVM32)	2,166	30.9
VM2(pVM33)	1,081	15.4
VM2(pVM34)	78	1.1
VM2(pVM35)	408	5.8

TABLE 4. Activation of the *ctx-lacZ* operon fusion by strains carrying *toxR* and *toxS* on separate plasmids

Strain	β-Galactosidase activity (U)	Activation
VM2	95	1.0
VM2(pVM34, pACYC184)	72	0.8
VM2(pVM34, pVM51)	317	3.3
VM2(pVM34, pVM52)	73	0.8
VM2(pVM7, pACYC184)	805	8.5
VM2(pVM7, pVM51)	856	9.0
VM2(pVM7, pVM52)	895	9.4

DNA sequence analysis showed that the deletion in 569B occurs within this *NcoI-BglII* fragment (V. J. DiRita and J. J. Mekalanos, manuscript in preparation). The only difference between pVM7 and pVM33 is the presence in pVM33 of the 1.2 kbp of DNA downstream of *toxR* from E7946 which is deleted in 569B. pVM7 and pVM33 activated *ctx* expression to the same (high) level (Table 3); therefore, the lower levels of toxin produced by E7946 cannot be explained by a negative effect on ToxR-mediated *ctx* expression encoded within the downstream 1.2 kbp of DNA.

In pVM7 and pVM33, the *toxR* gene is transcribed from the *tet* promoter of pBR322 (data not shown). Since these two plasmids are apparently independent of the downstream DNA for expression of ToxR activity, the possibility thus remained that the downstream DNA encodes a factor that interacts with *toxR* promoter sequences not present in these constructs. To test this possibility, several other plasmids were constructed. The *Eco*RI fragment of pVM7 and pVM33 (containing the promoter for the *tet* gene of pBR322 and encoding 52 N-terminal amino acids of *toxR*; 16) was replaced in each of these plasmids with the 670-bp *Eco*RI fragment of pVM49 which carries the E7946 *toxR* promoter as well as the N terminus of *toxR* (Fig. 1). Thus pVM34 and pVM35, derived from pVM7 and pVM33, respectively, contain most of the *toxR* gene of 569B, while the region upstream of *toxR* in both plasmids is derived from E7946. pVM34 failed to activate expression of *ctx*::*lacZ*, whereas pVM35 activated the *ctx* promoter to a level similar to that induced by the E7946 *toxR* plasmids pVM48 and pVM49 (Table 3). The only structural difference between pVM7 and pVM34 is that pVM34 has the promoter region from E7946. This result suggests either that the expression of the E7946 *toxR* promoter requires the region downstream of the *toxR* gene which is deleted in strain 569B or that ToxR requires a function encoded by the downstream DNA for its activity when expressed from the E7946 promoter.

To ensure that the inability of plasmid pVM34 to activate *ctx* expression was not due to an artifact of the 569B-E7946 hybrid *toxR* construction, most of the 1.2 kbp of DNA downstream of *toxR* was deleted from pVM48, the original E7946 *toxR* clone. This was accomplished by digesting pVM48 with *Hin*dIII and religating. This removes a 4.3-kbp *Hin*dIII fragment from pVM48, including all but 53 bp of the 1.2-kbp region, and the resulting plasmid, pVM50, is therefore similar to pVM34 (Fig. 1). pVM50 failed to activate *ctx*::*lacZ* even though the entire E7946 *toxR* gene and promoter region are intact (data not shown).

Evidence for a *trans*-acting function encoded downstream of *toxR*. The data presented above suggest that a function required for activation of *ctx* expression by ToxR is encoded within the 1.2 kbp of DNA downstream of *toxR* in E7946. To determine whether this DNA is capable of acting when present in *trans* with respect to *toxR*, plasmids pVM51 and pVM52 were constructed (Fig. 1). These plasmids carry a 3.2-kbp *Eco*RI fragment from pVM49 cloned in opposite orientations into the *Eco*RI site of pACYC184 (Fig. 1). This 3.2-kbp *Eco*RI fragment has the C-terminal coding sequences of ToxR in addition to the 1.2-kbp downstream activator region (16). pVM51 and pVM52 do not activate *ctx*; thus, they do not have an active *toxR* gene (data not shown). We transformed strains VM2(pVM7) and VM2(pVM34) with pVM51 and pVM52. The combination of pVM51 and pVM34 activated *ctx*::*lacZ* expression, whereas pVM34 with either pACYC184 or pVM52 did not (Table 4). Activation of *ctx* expression by pVM7 was unaffected by pVM51 and pVM52. This result indicates that the region downstream from *toxR* in E7946 contains a gene which encodes a *trans*-acting activator of *toxR* and which we call *toxS*. The fact that pVM51, and not pVM52, is ToxS$^+$ suggests that the *Eco*RI fragment containing the *toxS* gene in these plasmids lacks a functional promoter. Expression of DNA cloned into the *Eco*RI site of pACYC184 is from the promoter of the gene encoding chloramphenicol resistance (1).

ToxS may act by altering the expression of the *toxR* gene from the E7946 promoter (contained in the 670-bp *Eco*RI fragment of pVM34) since this promoter is required to see the effect (pVM7 is independent of ToxS). It is also possible that the *toxS* gene product activates the ToxR protein from E7946, but not that from 569B, by interacting specifically with an amino-terminal domain of the E7946 ToxR (within the 670-bp *Eco*RI fragment of pVM34; Fig. 1). This possibility is unlikely as the DNA sequences of the *toxR* genes from E7946 and 569B vary by a single nucleotide in this region. This change does not affect the predicted amino acid sequence of ToxR (data not shown; 16).

Analysis of a *toxR*::*lacZ* fusion plasmid; ToxS does not directly activate the *toxR* promoter. To test directly whether ToxS is capable of activating expression from the *toxR* promoter, a *toxR*::*lacZ* gene fusion plasmid was constructed (Fig. 2). This plasmid, pVJ20, contains the 530-bp *Eco*RI-*Eco*RV promoter fragment from pVM35 inserted in the *Eco*RI and *Sma*I sites of the *lacZ* gene fusion plasmid pMLB1010 (22). pMLB1010 expresses a relatively high background level of β-galactosidase, and insertion of the *toxR* promoter leads to a slight but reproducible increase of this activity (Table 5). We assayed the β-galactosidase activity from pVJ20 in combination with various *toxR*- and *toxS*-containing plasmids (Table 5). There was no activation (indeed, there was a slight depression) of *toxR*::*lacZ* expression from pVJ20 when this plasmid was carried together with either pVM25 (ToxR$^+$S$^-$) or pVJ21 (ToxR$^+$S$^+$). A similar lack of activation of *toxR*::*lacZ* encoded by pVJ20 was observed with either pVM51 (ToxR$^-$S$^+$) or pVM52 (ToxR$^-$S$^-$; data not shown). This result suggests that ToxS does not activate the *toxR* promoter and also seems to eliminate the possibility that ToxS causes ToxR to activate the *toxR* promoter.

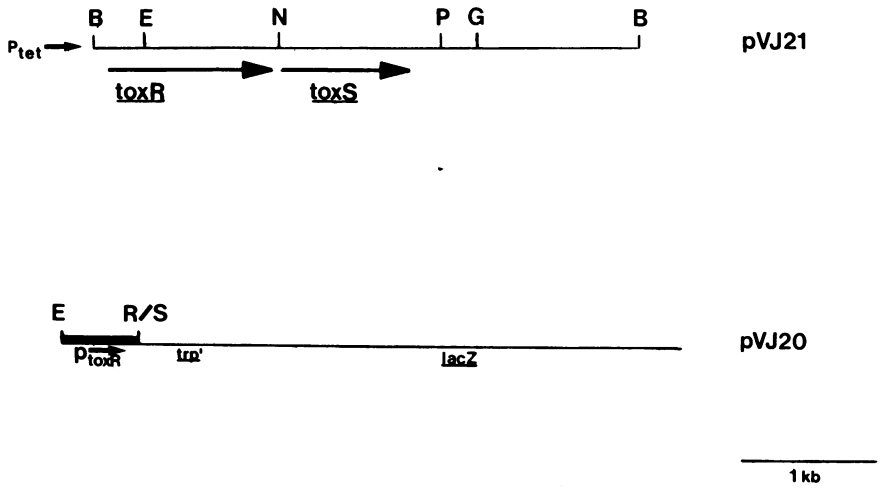

FIG. 2. Plasmids used to study the activation of the *toxR* promoter by ToxR and ToxS. pVJ21 consists of a 3.6-kb *Bam*HI fragment containing *toxR* and *toxS* cloned into the *Bam*HI site of pACYC184 (1). *toxR* and *toxS* are expressed from the *tet* promoter in this plasmid (data not shown). pVJ20 contains a 570-bp *Eco*RI-*Eco*RV fragment from plasmid pVM35 (Fig. 1), cloned into the *Eco*RI-*Sma*I sites of the promoterless *lacZ* vector pMLB1010 (22). This fragment contains the *toxR* promoter and 30 nucleotides of coding sequence from within the gene (16).

The *toxR* and *toxS* genes constitute an operon in *toxS*$^+$ strains of *V. cholerae*. As demonstrated above, pVM51, but not pVM52, is ToxS$^+$ (Table 4). This suggests that the *toxS*-containing fragment in these plasmids lacks a functional promoter and that in pVM51 the gene is expressed from the promoter for the chloramphenicol resistance gene of pACYC184 (1). The orientation of the E7946 DNA inserted in pVM51 (with respect to the plasmid promoter) suggests that *toxS* is transcribed from the same DNA strand as *toxR* (Fig. 1) and therefore may be part of the same transcriptional unit as *toxR*. If this is the case, then the *toxR* mRNA from 569B should be a different size from the *toxR* mRNA of strains that have *toxS*. Figure 3 shows a Northern blot of mRNA isolated from several *V. cholerae* strains probed with sequences internal to the *toxR* structural gene (16). Strains O395 and M7922 exhibited a different hybridization pattern than did 569B; in particular, they displayed *toxR*-homologous mRNA larger than that seen in 569B. This reinforces the suggestion that *toxR* and *toxS* are cotranscribed. Taking this with the observations that *toxS* is downstream of *toxR* and in the same transcriptional orientation, we conclude that *toxR* is promoter proximal in this transcript.

The multiple homologous mRNAs seen on the Northern blot may represent specific degradation products; this blot was used previously to analyze *ctx* mRNA (13) and showed no evidence of general RNA degradation. In addition, previous Southern blot analysis gave no indication of multiple *toxR* genes (13, 14). Other evidence that these fragments are derived from *toxR* comes from analysis of the *toxR* mutant strain M13, which synthesizes 1,000-fold less toxin than its 569B parent (7). This strain displayed greatly reduced hybridization to all RNA fragments (Fig. 3), indicating that the mutation in this strain probably affects the 569B *toxR* promoter or the stability of *toxR* mRNA.

TABLE 5. Effect of ToxR and ToxS on the expression of *toxR*::*lacZ*

Plasmid	β-Galactosidase activity (U)
pMLB1010	486
pVJ20 (*toxR*::*lacZ*)	702
pVJ20 + pACYC184	804
pVJ20 + pVM25 (ToxR$^+$S$^-$)	554
pVJ20 + pVJ21 (ToxR$^+$S$^+$)	519

DISCUSSION

In this report, we present evidence that the *toxS* gene lies directly downstream from *toxR* in the *V. cholerae* El Tor strain E7946 but is deleted in the classical strain 569B. That this gene is present in several other strains of *V. cholerae* was inferred from Southern blot analysis in which all strains studied displayed a restriction fragment that was 1.2 kbp larger than that displayed by strain 569B (14). We have demonstrated that *toxS* encodes an activity which acts in *trans* and is required for ToxR to activate *ctx* expression when the wild-type *V. cholerae toxR* promoter is used to express *toxR*. Our data suggest that ToxS does not directly activate the *toxR* promoter and that it does not cause ToxR to activate its own promoter. We infer from this that ToxS may act to modify ToxR in such a way as to make it competent for transcriptional activation of the *ctxAB* promoter.

The requirement for a second gene product by a bacterial transcriptional activator has been observed in several cases

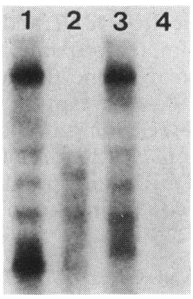

FIG. 3. Northern blot of *V. cholerae* strains, using a *toxR*-specific probe. The lanes contain RNA from the following *V. cholerae* strains: 1, O395; 2, 569B; 3, M7922; 4, M13.

in which there is a specific pattern of gene expression in response to an external stimulus. Regulation of expression of the genes involved in assimilation of nitrogen in *E. coli* is dependent upon the action of a sensor, NR_{II}, and a regulator, NR_I. Under conditions of nitrogen limitation, NR_{II} phosphorylates NR_I. Phosphorylated NR_I then activates transcription of the glutamine synthetase gene (17). Other pairs of genes which share a "sensor/regulator" relationship are *envZ/ompR*, *cpxA/sfrA*, and *phoR/phoB* in *E. coli* and *virA/virG* in the phytopathogen *Agrobacterium tumefaciens* (21, 24, 25). ToxR shares amino acid similarity with the OmpR subclass of these regulators (16), but differs from the others by being a transmembrane protein. It has been suggested that ToxR is a one-component system analogous to the two-component regulatory systems (21), but the data presented in this report indicate that ToxR does not act alone in transcriptional activation.

The strain from which *toxR* was originally cloned, 569B, was isolated as a toxin-overproducing derivative after serial animal passage of a virulence-attenuated strain (2). Strain 569B expresses ToxR-regulated genes under in vitro growth conditions (pH 8.5) which inhibit such expression in other strains (J. Mekalanos, unpublished data). Perhaps the virulence-attenuated strain which was the parent of 569B was a *toxS* deletion mutant, and animal passage led to selection of a strain in which ToxR is independent of ToxS. We have recently isolated a spontaneous mutant of the classical strain O395-N1 which carries a *toxS* deletion similar to that observed in 569B (Mekalanos, unpublished data). This strain, JJM42, does not express ToxR-regulated genes under the conditions in which they are expressed by 569B. In addition, JJM42 has the altered outer membrane protein profile characteristic of ToxR mutants and does not undergo TCP-mediated in vitro autoagglutination (15, 26). This phenotype can be reverted by introduction of *toxS* into this strain (DiRita and Mekalanos, in preparation).

The reason for the ToxS independence of 569B is not clear at this time. Experiments using hybrid plasmid constructions (Table 3) demonstrated that there is no substantial difference in the *toxR* coding information in strains 569B and E7946. DNA sequence analysis revealed differences between the E7946 and 569B *toxR* genes which would account only for very conservative amino acid changes between ToxR proteins from 569B and E7946 (DiRita and Mekalanos, in preparation). One possibility is that ToxR may become independent of ToxS when the *toxR* gene is expressed from a stronger promoter. To date, we do not know the structure of the *toxR* promoter from either E7946 or 569B. That ToxR could become independent of ToxS when overexpressed is analogous to the observation that, in *E. coli*, elevated expression of the transcriptional activator OmpR obviates its requirement for EnvZ (23).

Finally, we conclude that *toxR* and *toxS* constitute an operon (with *toxR* promoter proximal) because restriction fragments containing *toxS* alone do not express the gene unless they are cloned downstream of a vector promoter in the same orientation as the coding strand of *toxR*. Consistent with this interpretation is Northern blot analysis in which a *toxR* probe displays an mRNA in 569B which is smaller than that displayed in *toxS*-containing strains of *V. cholerae*.

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