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Genetics

Synthesis of cholera toxin is positively regulated at the transcriptional level by *toxR*

(molecular cloning/*lacZ* fusion/genetics of virulence)

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ABSTRACT **We have cloned a positive regulatory gene (*toxR*) from *Vibrio cholerae* that controls cholera toxin transcription. This was done by first constructing a genetic fusion consisting of the *lacZ* gene fused to the promoter of the cholera toxin operon *ctxAB*. This operon fusion was used to screen a *V. cholerae* genomic library for genes that could activate the *ctx* promoter in *Escherichia coli*. This method allowed the identification of a gene, *toxR*, that increases *ctx* expression by more than 100-fold. Complementation analysis indicated that certain hypotoxinogenic mutants of *V. cholerae* 569B probably have mutations in the *toxR* gene. Southern blot analysis suggests that all *V. cholerae*, including nontoxinogenic strains, have the *toxR* gene. Moreover, nontoxinogenic strains not only lack the structural genes for cholera toxin but also sequences associated with the larger 7-kilobase *ctx* genetic element.**

The diarrheal disease Asiatic cholera is induced by cholera toxin, a heat-labile protein produced by toxinogenic strains of *Vibrio cholerae* (1). Each toxin molecule is composed of two types of subunits, A (27 kilodaltons) and B (11.7 kilodaltons), encoded by the genes *ctxA* and *ctxB*, respectively (2, 3). The *ctxAB* operon shows genetic linkage to the *nal* region of the *V. cholerae* chromosome in strains of both the classical and the El Tor biotypes (4). All *V. cholerae* strains of the classical biotype thus far examined carry a structurally similar duplication of the *ctxAB* operon (5). Although most strains of the El Tor biotype possess only a single toxin operon copy, there are also El Tor strains that have two or more *ctxAB* copies (5), as well as other *V. cholerae* strains that have no detectable toxin gene sequences (6). This variation in the toxin operon copy number, together with recent physical data on the structure of *ctx* duplications (3, 5), suggests that the *ctx* genes reside on a larger genetic element that may undergo duplication, amplification, and transposition events. Thus, the study of cholera toxin regulation offers a system in which to analyze regulatory systems controlling the expression of virulence genes located on accessory genetic elements (e.g., transposons, phages, and plasmids).

Previous studies focusing on toxin regulation in *V. cholerae* have identified at least two different genetic loci that control toxin production in the classical strain 569B (7, 8). Mutations in a locus called *tox* cause a decrease in toxin production by a factor of 1000. Since strain 569B carries a duplication of the *ctx* operon (2, 5), this result suggests that the *tox* gene product is required for high expression of both *ctx* copies of this strain.

The two *ctx* operon copies from strain 569B have been cloned in *Escherichia coli*, but synthesize 100-fold more B subunit in *V. cholerae* than in *E. coli* (3). While it is possible that the *ctx* transcriptional and translational signals are not recognized efficiently by the *E. coli* synthetic machinery, an alternative explanation for this difference, consistent with known *V. cholerae* regulatory mutations, is that *E. coli* lacks the *V. cholerae* regulatory gene defined by mutations in the *tox* locus.

Here we present data concerning the cloning of a *V. cholerae* gene, *toxR*, that increases expression of *ctx* in *E. coli*. RNA blot analysis and expression of *ctx–lacZ* operon fusions indicates that *toxR* is a positive regulator of *ctx*, and that it acts at the transcriptional level. Complementation of *tox* mutations by *toxR* indicates that *toxR* may be identical to the previously described *tox* locus.

MATERIALS AND METHODS

Bacterial Strains and Phage Stocks. *V. cholerae* and *E. coli* strains were maintained at −70°C in LB medium (9) containing 25% (vol/vol) glycerol or on LB plates. Antibiotics were used at the following concentrations: ampicillin (Ap), 50 μg/ml; tetracycline (Tc), 15 μg/ml; kanamycin (Km), 30 μg/ml. Phage λ NF1955 and its derivatives were grown and purified as described (10).

Nucleic Acid Preparation and Analysis. Chromosomal and plasmid DNA was purified as described (5). CT-1, L-1, and L-3 probes were prepared and labeled as described (5). A *toxR* probe was prepared by T4 DNA polymerase replacement synthesis (10) of plasmid pVM15 digested with *Bam*HI. Plasmid pVM15 is a derivative of pJM22 (3) that carries a 2.4-kilobase (kb) *Bam*HI fragment containing *toxR*. Southern blot analysis was carried out under high stringency as described (5). DNA restriction enzymes and phage T4 DNA ligase were purchased from New England Biolabs.

RNA for RNA blot analysis was extracted from late logarithmic phase cells grown in CYE medium (11) at 30°C using the hot phenol method essentially as described by Aiba *et al.* (12). Thirty micrograms of RNA was loaded per lane, electrophoresed in a denaturing gel, transferred to nitrocellulose, and hybridized with a labeled probe as described by Godowski and Knipe (13) with minor modifications. The baked nitrocellulose was rehydrated in 0.15 M NaCl/0.015 M Na citrate (1× NaCl/Cit) (13), and prehybridized with 10 ml of hybridization solution (50% formamide/5× NaCl/Cit/50 mM KPO_4 buffer, pH 6.5/0.02% Denhardt's solution/0.25% Na$DodSO_4$) at 37°C for 24 hr. The prehybridization solution was removed and 3 ml of hybridization solution containing heat-denatured calf thymus DNA at 250 μg/ml and CT-1 probe was added. After hybridization at 42°C for 48 hr, the filter was washed in 1 liter of 5× NaCl/Cit/0.1% Na$DodSO_4$ at 55°C with gentle shaking for 20 min. This was followed by washes with 800 ml and 200 ml of the same solution at 55°C for 15 min and 5 min, respectively. The filter was then washed in 500 ml of 4× NaCl/Cit at ambient temperature for 10 min, followed by a wash in 500 ml of 2× NaCl/Cit for 10 min. The filter was air dried and autoradiographed.

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Abbreviations: kb, kilobase(s); NaCl/Cit, standard saline citrate.
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Toxin and β-Galactosidase Assays. Toxin antigen was quantitated by a GM1-dependent, enzyme-linked immunosorbent assay (14) in *V. cholerae* culture supernatants or in extracts of *E. coli*, grown to late exponential phase at 30°C with shaking as described. β-Galactosidase activity was assayed as detailed by Miller (9).

Construction of a Phage, λ NFVM1, Carrying the *ctx–lacZ* Operon Fusion. The operon fusion vector pMLB1010 (15) was used to construct a *ctx–lacZ* operon fusion as follows: A 2.7-kb *Eco*RI/*Xba* I fragment containing the *ctx* promoter and coding sequence for the NH_2-terminal 28 amino acid residues of the A subunit was obtained from plasmid pJM23.2 (3) and inserted into the *Eco*RI of pMLB1010 to give pVM26. A 2.9-kb *Nde* I/*Eco*RI fragment coding for part of *ctxB* and also containing an insertion encoding Km resistance, was inserted at a *Bal* I site downstream of the *lacZ* gene of pVM26 to give plasmid pVM27 (Fig. 1). λ NF1955 (*cI857 Sam100 int*$^+$ *att*$^+$) contains a unique *Eco*RI site and sequences coding for the carboxyl terminus of *lacZ* (15). This phage forms white plaques on LB plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XG) at 40 μg/ml when propagated on a *lac*$^-$ strain. Phage λ NFVM1 was constructed as follows (see Fig. 1): 1.0 μg of purified λ NF1955 DNA and 1.0 μg of pVM27 were digested with *Eco*RI, ligated with T4 DNA ligase for 4 hr at 18°C, then ethanol precipitated and resuspended in 10 μl of 10 mM Tris·HCl, pH 8.0/1 mM EDTA. The ligation mix was then packaged *in vitro* and the resulting phage were plated on *E. coli* strain XAC*suIII* [Δ (*lac pro*) *ara*$^-$ *argEam nalA rif*$^-$ *thi metB*$^-$ *suIII*] as described (10). Homologous recombination *in vivo* between regions of *lacZ* homology resulted in deletion of the *ctxB* and Km^r sequences (indicated by the dotted box in Fig. 1). Blue plaques were purified and used to lysogenize strain MS327 [*F*$^-$ *araD* Δ(*lac pro*) *argEam rif*^{r} *nalA recA56*]. These lysogens formed colonies that were Km^s and were pale blue on plates containing XG. Southern blot analysis confirmed the presence and structure of the *ctx–lacZ* operon fusion on the chromosome (data not shown). One such lysogen, VM2, was used for subsequent experiments.

RESULTS

RNA Blot Analysis of *ctx* mRNA Production. The phenotype of *tox* mutants of strain 569B suggests that they carry regulatory mutations that affect the expression of the toxin structural genes (7). To determine whether *tox* mutations affect the level of *ctx* mRNA, we performed RNA blot analysis of mRNA synthesized by a wild-type 569B strain, several *tox* mutants, and M7922, a *ctx* deletion mutant of strain RV79 (16). As can be seen in Fig. 2, M7922 and the *tox* mutants show no detectable RNA capable of hybridizing to a *ctx*-specific gene probe, CT-1. This result suggests that the *tox* gene product acts at the transcriptional level. Since *tox* mutations occur with relatively high frequency (>0.1%) after nitrosoguanidine mutagenesis (17–19), these data also suggest that *tox* mutations probably inactivate a positive regulatory element required for high expression of the *ctx* promoter.

Molecular Cloning of the *toxR* Gene. Given the likelihood that the *tox* gene product acts at the transcriptional level, we reasoned that it might be possible to clone the *tox* gene of *V. cholerae* by screening for increased transcriptional activity from the *ctx* promoter. To do this, we constructed *in vitro* an operon fusion of the *ctx* promoter to the structural gene for β-galactosidase, *lacZ* (Fig. 1). The *ctx–lacZ* fusion was then inserted into the cloning vector λ NF1955 to give λ NFVM1. *E. coli* strain MS327 (Δ *lac*) was lysogenized with λ NFVM1 to give strain VM2, which therefore contains a copy of the *ctx–lacZ* fusion on its chromosome. Thus, β-galactosidase produced by VM2 should reflect the amount of transcription originating from the *ctx* promoter (20, 21). VM2 was transformed with a plasmid library consisting of *Sau*3A partially digested DNA fragments from strain 569B cloned into the *Bam*HI site of pBR327. The resultant colonies were screened on LB plates containing XG and Ap. Since VM2 is pale blue on XG plates, colonies with recombinant plasmids carrying a gene that activates the *ctx* promoter were expected to be dark blue on XG plates. Two such colonies out of ≈5,000 screened were found to have this phenotype. The recombinant plasmids purified from these two colonies were named pVM6 and pVM8. Retransformation of VM2 with pVM6 or pVM8 confirmed that this phenotype was a plasmid-linked trait. In addition, transformation of these plasmids into MS327 gave white colonies, indicating the *ctx–lacZ* fusion carried by VM2 was the source of the β-galactosidase seen in the dark blue plasmid-carrying colonies.

Restriction enzyme analysis of the two plasmids, pVM6 and pVM8, showed that they carry *V. cholerae* DNA inserts of 8.4 kb and 6.5 kb, respectively. These inserts have a num-

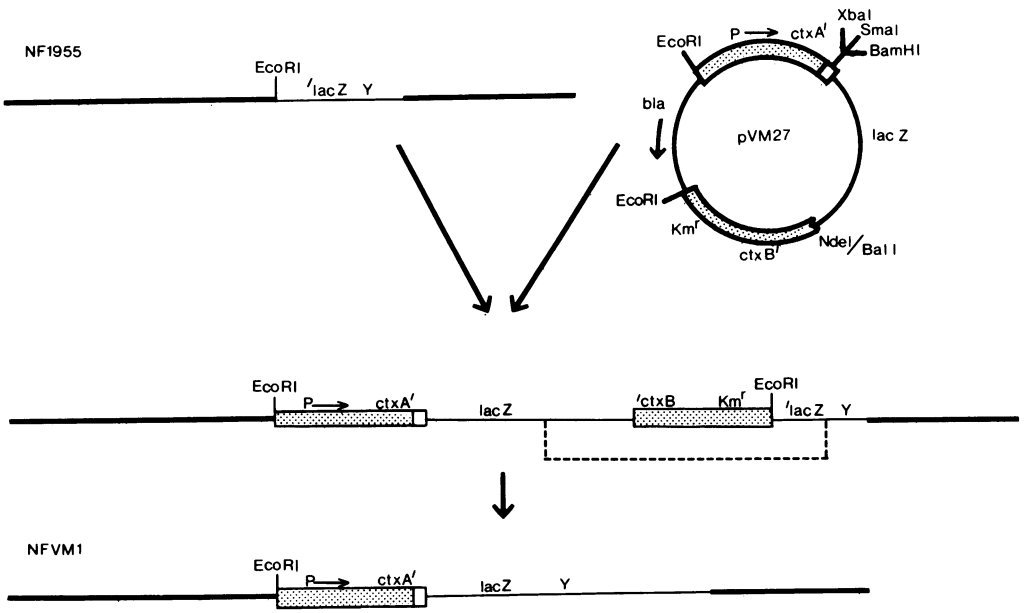

FIG. 1. Construction of a λ phage carrying the *ctx–lacZ* operon fusion.

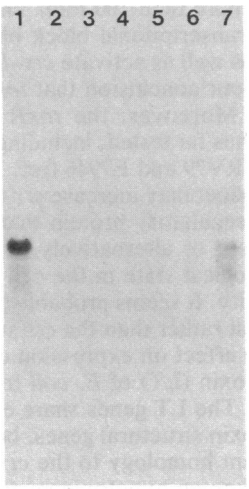

FIG. 2. RNA blot analysis of *ctx* mRNA production. RNA was purified from strain 569B; 569B *tox* mutants M13, MN1, RM6, and RM3; and *ctx* deletion mutant M7922. The RNA was fractionated by electrophoresis in agarose gels, transferred to nitrocellulose, and probed with CT-1 probe. The lanes contain RNA from the following *V. cholerae* strains: 1, 569B (laboratory collection); 2, M7922 (16); 3, M13 (17); 4, MN1 (8); 5, RM6 (18); 6, RM3 (18); 7, RM3 pVM7.

ber of restriction fragments in common, indicating that we had obtained two independent clones of the same *V. cholerae* gene. Subcloning experiments showed that the gene responsible for the activation of the *ctx–lacZ* fusion is located on a 2.4-kb *Bam*HI fragment present on both pVM6 and pVM8. This fragment was inserted at the *Bam*HI site of pBR322 to give plasmid pVM7.

Table 1 shows that pVM7 activates the *ctx–lacZ* fusion of VM2 13-fold. To confirm that pVM7 increased β-galactosidase production by activation of the toxin promoter, we examined the effect of pVM7 on expression of *ctxB* carried by plasmid pJM290.2 (3). The latter plasmid carries an internal *ctxA* deletion mutation, but it continues to express *ctxB* via the natural *ctx* promoter. As shown in Table 1, pVM7 activates *ctxB* expression more than 100-fold. A simple model explaining these results is that pVM7 carries a toxin regulatory gene, which we call *toxR*, that activates transcription from the *ctx* promoter.

Complementation Analysis with the Cloned *toxR* Gene. Since *toxR* exhibits the phenotype expected of the wild type *tox* locus (7), we tested the ability of *toxR* to complement *V. cholerae tox* mutants. This was done by mobilizing pVM7 into these mutants and then scoring the resultant strains for toxin expression. From the results shown in Table 2, it is clear that pVM7 suppresses the hypotoxinogenic phenotype of these mutants by a factor of at least 300 to 1000 to give wild-type levels of B-subunit production. The fact that

Table 1. Activation of the *ctxAB* promoter by pVM7

Strain	β-Galactosidase activity	B subunit
VM2	69	ND
VM2 pBR322	78	ND
VM2 pVM7	880	ND
VM2 pJM290.2	70	0.002
VM2 pJM290.2 pBR322	68	0.002
VM2 pJM290.2 pVM7	825	0.29

E. coli grown to late exponential phase in LB with shaking at 30°C was used for β-galactosidase (9), and B-subunit assays (14). Purified toxin was used as a standard (11). Values are reported as μg of toxin antigen equivalents per ml of culture. ND, not detectable (less than 0.001 μg/ml).

Table 2. Effect of pVM7 on expression of *ctxAB* in *tox* mutants and wild-type *V. cholerae*

Strain	*ctx tox* allele	B-subunit production: No plasmid	B-subunit production: With pVM7	Ref.
M13	*ctxA*$^+$ *B*$^+$ *tox*-2	ND	0.33	17
RM3*	*ctxA*$^+$ *B*$^+$ *tox*-302	ND	0.33	18
RM4†	*ctxA*$^+$ *B*$^+$ *tox*-303	ND	1.0	18
RM6	*ctxA*$^+$ *B*$^+$ *tox*-305	ND	1.0	18
RM8	*ctxA*$^+$ *B*$^+$ *tox*-304	ND	0.33	‡
MN1	*ctxA*$^+$ *B*$^+$ *tox*-TI101	ND	0.33	8
M7922	Δ*ctxAB tox*$^+$	ND	ND	16
569B	*ctxA*$^+$ *B*$^+$ *tox*$^+$	1.0	0.33	§
RV79	*ctxA*$^+$ *B*$^+$ *tox*$^+$	0.004	0.01	§
SM201	*ctxA23 B*$^+$ *tox*$^+$	0.04	0.11	4

Transfer of pVM7 into *V. cholerae* strains was performed as described (3). Cultures were grown in CYE, or CYE with Ap, for 25 hr at 30°C with shaking. The supernatants were collected and used directly to determine B-subunit production. ND, not detectable (less than 0.001 μg/ml).
*Formerly RV501 Ltx-2.
†Formerly RV501 Ltx-3.
‡Unpublished data.
§Laboratory collection.

pVM7 had no stimulatory effect on toxin production in the parental 569B strain indicates that the *toxR* gene product is probably produced in sufficient quantity by the chromosomal copy of the locus to give maximal *ctx* activation. This result also argues against the possibility that *toxR* causes a nonspecific induction of toxin synthesis. It is also apparent that pVM7 does not carry the toxin structural genes, because the plasmid does not alter the negative phenotype of *ctx* deletion mutant M7922 (Table 2). RNA blot data, shown in Fig. 2, indicate that the *tox* mutant strain RM3 carrying pVM7 now produces RNA that hybridizes to DNA sequences specific for *ctxAB*.

Effect of *toxR* on *ctx* Expression in an El Tor Strain of *V. cholerae*. RV79 is an El Tor strain of *V. cholerae* that had a duplication of *ctxAB* (4, 5), yet produces about 1/100th as much toxin as 569B. RNA blots show that the low toxin production of RV79, and several other *V. cholerae* strains less toxinogenic than 569B, is reflected by lower levels of *ctx* mRNA (data not shown). Introduction of pVM7 into RV79 causes only a 2.5-fold increase in B-subunit production (Table 2). This suggests that the difference in expression between RV79 and 569B may reflect other regulatory differences between these two strains. For example, a variation in the *ctx* promoter sequences of RV79 and 569B may be responsible for this difference. This hypothesis was tested directly with strain SM201, which has the two resident *ctx* genes of RV79 replaced by a single copy of a *ctx* promoter and *ctxB* gene from 569B (4). As shown in Table 2, SM201 synthesizes ≈10-fold more B subunit than RV79, suggesting that the *ctx* operon of 569B is about 20 times more active than the *ctx* operon of RV79. The proposed *ctx* promoter regions of strains 569B and RV79 have been sequenced and show only one difference (3). A short sequence, T-T-T-T-G-A-T, located 77 base pairs upstream of the start of *ctxA* was found tandemly repeated 8 times for 569B-derived *ctx* copies but only 3 times for *ctx* copies derived from RV79. This repetitive sequence may, therefore, play a role in *ctx* regulation and could conceivably be interacting with the *toxR* gene product.

Southern Blot Analysis of *V. cholerae* Toxinogenic and Nontoxinogenic Strains. Southern blot analysis using the cloned *toxR* gene as probe indicates that all *V. cholerae* strains of the classical biotype thus far examined contain *toxR* sequences (data not shown). Therefore, it was of interest to examine strains of the El Tor biotype as well as nontoxino-

genic *V. cholerae* for *toxR* sequences. As shown in Fig. 3 the *toxR* probe hybridizes to a 3.9-kb *Eco*RI fragment in all these strains. Although the exact location of *toxR* sequences on the *toxR* probe used here is not known, the fact that the probe hybridizes with similar efficiency to the same small *Eco*RI fragment in both wild-type and nontoxinogenic strains, strongly suggests that this *Eco*RI fragment contains sequences homologous to the *toxR* gene. Moreover, genetic evidence to be presented elsewhere indicates that these *toxR* homologous sequences are associated with a functional *toxR* gene (unpublished data). Thus, these data suggest that an intact regulatory system for *ctx* genes is present in naturally occurring nontoxinogenic strains of *V. cholerae* as well as in toxinogenic strains of both biotypes.

Structural analysis of the *ctx* region indicates that *ctxAB* is part of a 7-kb genetic element that is amplifiable and has structural features analogous to transposons (5). Two DNA probes, L-1 (a 2.7-kb *Pst* I/*Xba* I fragment) and L-3 (a 2.7-kb *Bgl* II/*Bgl* II fragment), define regions of the *ctx* genetic element located upstream of the *ctx* operon. Fig. 3 shows that nontoxinogenic strains not only lack *ctxAB* sequences but also sequences homologous to the L-1 and L-3 probes. Therefore, it appears that nontoxinogenic strains of *V. cholerae* lack the entire *ctx* genetic element yet retain the *toxR* regulatory system. Thus, these nontoxinogenic strains might serve as a potential reservoir of fully virulent *V. cholerae* after acquisition of the *ctx* genes and other potential virulence genes associated with the *ctx* genetic element.

DISCUSSION

We have presented evidence that the cholera toxin operon *ctxAB* is positively regulated at the transcriptional level by the *V. cholerae* gene *toxR*. We cloned the *toxR* gene by screening a *V. cholerae* genomic library for plasmids capable of activating a *ctx–lacZ* operon fusion in *E. coli*. The cloned *toxR* gene increases *ctx* expression in *E. coli* and in *V. cholerae tox* mutants more than 100-fold. The ability of *toxR* to alleviate the *ctx* transcriptional block observed in *V. cholerae tox* mutants as well as activate *ctx–lacZ* operon fusions strongly supports our conclusion that *toxR* acts at the transcriptional level. Moreover, the *toxR* gene activates all cloned *ctx* genes thus far tested, including those cloned from the El Tor strains RV79 and E7946 (ref. 3; data not shown). The *toxR* gene product may increase *ctx* transcription by encoding a positive regulatory protein that interacts with the *ctx* promoter region, or alternatively, it may simply act to produce a physiological state in the cell necessary for high *ctx* promoter activity. It seems probable that *toxR* acts at the *ctx* promoter region rather than the *ctx* structural genes, because *toxR* has no effect on expression of the genes for the heat-labile enterotoxin (LT) of *E. coli* (refs. 24 and 25; unpublished results). The LT genes share extensive homology with the cholera toxin structural genes, but the LT promoter shows no significant homology to the *ctx* promoter (3).

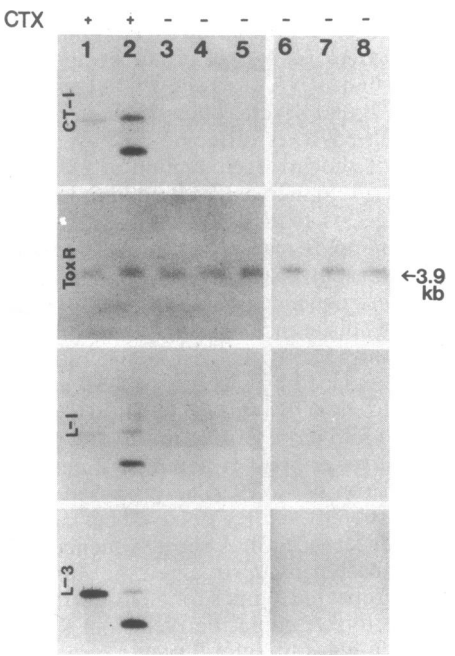

FIG. 3. Southern blot analysis of *V. cholerae* toxinogenic and nontoxinogenic strains. DNA was purified from toxinogenic and nontoxinogenic *V. cholerae*, digested with *Eco*RI, fractionated by electrophoresis in agarose gels, transferred to nitrocellulose, and hybridized with either CT-1, ToxR, L-1, or L-3 probes. The lanes contain DNA from the following *V. cholerae* strains: 1, 3083 (19); 2, 2560-78 (22); 3, 2740-80 (22); 4, 2843-80A (22); 5, M7922 (16); 6, 1074 (23); 7, 1196 (23); 8, Nag-1 (provided by P. Echeverria).

Suppression of known hypotoxinogenic *tox* mutations by *toxR* to give wild-type levels of toxin production indicates that *tox* mutant strains such as M13 (7, 17, 19) probably carry mutations in the *toxR* gene. The fact that the M13 mutant induced no diarrheal syndrome in human volunteers (26) underlines the important role that the *toxR* gene must play in the regulation of cholera toxin production in the pathogenic environment of *V. cholerae*—i.e., the upper intestine of man.

Although mutations strictly analogous to the *tox* mutations of 569B have not been described in other classical and El Tor strains of *V. cholerae*, several lines of evidence suggest that these strains also contain *toxR*. First, Southern blot analysis indicates that the genomes of all El Tor and other classical strains thus far examined contain sequences homologous to the cloned DNA fragment carrying the *toxR* gene when probed under conditions of high stringency (Fig. 3; data not shown). Second, the *ctx* genes from strain 569B are expressed at a high level in M7922, a *ctx* deletion mutant of the El Tor strain RV79 (3, 16). If M7922 did not contain a functional *toxR* gene, one might expect levels of *ctx* expression analogous to *tox* mutants of 569B. Finally, in conjugative crosses, RV79 can donate a chromosomal locus to 569B *tox* mutants that both suppresses their hypotoxinogenic phenotype and also displays the characteristic genetic linkage of the *tox* locus of strain 569B (unpublished results). Together, these data support the conclusion that *toxR* is both present and active in other strains of *V. cholerae* besides 569B. The factors that control the production of the *toxR* gene product or its interaction with the product of the other toxin regulatory locus, *htx* (8), are currently under investigation.

Several years ago a negative transcriptional regulatory system for diphtheria toxin was described. Diphtheria toxin is encoded by a temperate bacteriophage called β, which infects and lysogenizes *Corynebacterium diphtheriae* (27, 28). It has been proposed by Murphy and co-workers (29–31) that diphtheria toxin synthesis is regulated at the transcriptional level by a repressor-like bacterial factor that responds to the concentration of iron in the growth medium. Both phage and bacterial mutations have been isolated that affect toxin regulation in ways predicted by this model (32, 33). In contrast to the regulation of diphtheria toxin, cholera toxin appears to be positively regulated at the level of transcription. However, like diphtheria toxin, cholera toxin synthesis does respond to nutritional signals (34–39), and this response may well be mediated by the *toxR* gene product. A further analogy between diphtheria toxin and cholera toxin can be drawn. Nonlysogenic *C. diphtheriae* has a regulatory system for diphtheria toxin genes it may acquire from phage β. Our Southern blot analysis suggests that nontoxinogenic strains of *V. cholerae* lack the entire *ctx* genetic element but still contain *toxR* gene sequences. If these *toxR* homologous se-

quences are indeed active, then it would seem that nontoxinogenic *V. cholerae* strains present in the environment also have a regulatory system for cholera toxin genes they might acquire in nature. What the function of either of these regulatory systems is in the absence of their respective toxin genes is unknown. However, their presence in nontoxinogenic strains raises interesting questions as to the evolutionary origins of these regulatory genes as well as the toxin genes they control.

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