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Genetics

Regulatory cascade controls virulence in *Vibrio cholerae*

(pathogenesis/coordinate regulation/transcriptional activation/ToxR)

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ABSTRACT **Expression of more than 17 virulence genes in *Vibrio cholerae* is under the coordinate control of the ToxR protein. ToxR is a transmembrane protein that binds to and activates the promoter of the operon encoding cholera toxin. As yet, the ability of ToxR to activate directly other genes in this regulon has not been demonstrated. We have cloned a gene called *toxT* from *V. cholerae* 569B; the *toxT* gene product, like ToxR, can activate the *ctx* promoter in *Escherichia coli*. In addition, expression of other genes identified as members of the ToxR regulon (*tcpA*, *tcpI*, *aldA*, and *tagA*) can be activated in *E. coli* by the *toxT* gene product but not by ToxR. When expressed from a constitutive promoter, the *toxT* gene product partially suppresses the ToxR$^-$ phenotype of a *toxR* deletion mutant of *V. cholerae*. The level of toxT mRNA is greatly reduced in a *toxR* mutant of *V. cholerae*. In addition, growth conditions under which the ToxR regulon is not expressed also repress the synthesis of toxT mRNA. These results suggest that ToxR controls transcription of *toxT*, whose product in turn is directly responsible for activation of several virulence genes under ToxR control.**

Vibrio cholerae is the etiologic agent of the diarrheal disease cholera. In response to specific environmental conditions, this organism expresses several virulence determinants including the cholera toxin (Ctx), a toxin-coregulated pilus (Tcp), and accessory colonization factor (Acf; refs. 1–3). These gene products are part of a regulon under the control of the products of the *toxRS* locus of *V. cholerae* (4–6). ToxR is a transmembrane protein that binds specifically to the promoter for the operon encoding the cholera toxin (*ctxAB*) and activates transcription from this promoter in *Escherichia coli* (4, 7).

Seventeen ToxR-activated genes (*tag* genes) have been defined by screening Tn*phoA* fusions whose expression is coordinately regulated with that of cholera toxin (3). Most of the *tag* genes have been defined as being involved in Tcp and Acf biogenesis (3). In addition, the expression of two major outer membrane proteins, OmpU and OmpT, is controlled by ToxR (8). Unlike *ctxAB*, the *tag* genes analyzed so far are not activated by ToxR in *E. coli* (C.P. and J.J.M., unpublished results) even though their expression is dependent on an intact *toxR* locus in *V. cholerae* (3). This has led us to the conclusion that there may be other transcriptional activators responsible for the expression of genes in the ToxR regulon and that these activators might themselves be under the control of ToxR (1). Such a "cascade" has been observed in sporulation by *Bacillus subtilis* where σ factors that regulate endospore formation appear in sequential fashion as they are required for developmental gene expression (9). A regulatory cascade system like this is thought to be an efficient way to control expression of sets of genes during different stages of development (9).

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In this report we present the cloning of the *toxT* gene from *V. cholerae* 569B. Like ToxR, the *toxT* gene product can activate the *ctx* promoter in *E. coli*. When expressed from a vector promoter, ToxT can direct coordinate expression of Ctx and Tcp in *V. cholerae* even in the absence of ToxR. Furthermore, several genes whose expression is dependent on ToxR in *V. cholerae* can be activated in *E. coli* by ToxT but not by ToxR. Consistent with the model of a regulatory cascade operating in *V. cholerae* virulence, we show that expression of *toxT* is controlled at the transcriptional level by ToxR.

MATERIALS AND METHODS

Bacterial Strains and Genetic Methods. Table 1 lists *V. cholerae* and *E. coli* strains used in this study. Strains were maintained at −70°C in LB medium (15) containing 20% (vol/vol) glycerol. A library of *V. cholerae* 569B in the plasmid pBR327 (4) was used as the source of clones from which the *toxT* gene was identified. The *tagA*::Tn*phoA* fusion was cloned from *V. cholerae* KP8.56, which was derived from strain O395 (3). *V. cholerae* JJM43 carries an internal deletion in the *toxR* gene and is a derivative of strain O395 (2, 14). Construction of the *toxR* insertion mutants O395-55 and 569B-55 and complementation with the ToxR$^+$ plasmid pVM53-D have been described (7, 8).

Transfer of IncP plasmids to *V. cholerae* was done by triparental mating using the mobilizing plasmid pRK2013 (16), as described (17).

Isolation and manipulation of plasmid DNA for cloning and analysis were done using standard protocols (18).

Biochemical Analyses. Assays for β-galactosidase and alkaline phosphatase were done using overnight cultures as described (2, 15). Analysis of Tcp pilus on Western blots was as described by Peterson and Mekalanos (3).

Cholera toxin production was assayed by a GM1-ELISA (19) of *V. cholerae* supernatants using polyclonal rabbit serum directed against purified cholera toxin. Toxin production is expressed in μg of toxin per culture OD_{600} unit.

Isolation of mRNA from *V. cholerae* was by the hot-phenol method (20). mRNA (5–10 μg) was subjected to electrophoresis on formaldehyde denaturing gels and Northern blots were done as described (18). Blots were probed with nick-translated restriction fragments purified from agarose gels (18). Conditions for probing and washing of blots were as described (21). Northern blots were stained with methylene blue to ensure that equal amounts of RNA were loaded in each lane (18).

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Table 1. Strains and plasmids used in this work

Strain or plasmid	Description	Ref. or source
E. coli		
SY327	F-Δ(*lac pro*) *nalA recA56 araD argE*	10
CC118	(*ara leu*)7697 *lacX74 araD139 phoA20 galE galK thi rpsE rpoB argE recA1*	11
DH5	F-Ò80d*lacZ* M15 (*lacZYA argF*)U169 *endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*	12
SK272	F-Δ*lacX74 galE galK thi rpsL phoA*	13
VM2	SY327 (λ NFVM1 *ctx*::*lacZ*)	4
V. cholerae		
O395	Prototrophic; Str^r	Laboratory collection
569B	Prototrophic; Str^r	Laboratory collection
O395-55	O395*toxR55*	8
569B-55	569B*toxR55*	8
JJM43	O395Δ*ctxA1*Δ*toxR43*	14
Plasmid		
pGJ40	pBR327 with *toxT* insert; Ap^r	This work
pGJ2.3	Replicon fusion pLAFR2::pGJ40; IncP Ap^s Tc^r	This work
pVC115	pLAFR2 with *tagA*::*phoA*/*aldA*::*lacZ* insert; Tc^r	C.P. and J.J.M.
pCS2.1	pLAFR2 with *tcpA*::*phoA* insert Tc^r Km^r	R. Taylor
pCS10.1	pLAFR2 with *tcpC*::*phoA* insert Tc^r Km^r	R. Taylor
pCS2.4	pLAFR2 with *tcpI*::*phoA* insert Tc^r Km^r	R. Taylor
pVM16	pBR322 with *toxR* insert Ap^r	5
pVM25	pACYC184 with *toxR* insert Cm^r	5

Str^r, streptomycin resistant; Ap^r, ampicillin resistant; Tc^r, tetracyclin resistant; Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant.

RESULTS

***toxR* Mutants of *V. cholerae* 569B Maintain Virulence Gene Expression.** Expression of virulence factors such as Ctx and Tcp is modulated in most strains of *V. cholerae* in response to environmental conditions and is dependent on the product of the *toxR* gene (2, 8). The classical strain 569B, however, does not modulate Ctx expression to the same degree as other strains in response to changes in the growth environment although it does contain an active *toxR* gene (4). Strain 569B carries a deletion of the *toxS* gene, whose product enhances the activity of ToxR in *E. coli* and *V. cholerae* (5, 7). That 569B is able to express ToxR-regulated genes without ToxS suggests that control of the ToxR regulon is altered in this strain relative to other strains of *V. cholerae*.

This was confirmed by comparing protein profiles of *toxR* mutants derived from strains 569B and O395. As shown in Fig. 1, a typical Coomassie-stained gel profile of strain O395 displays the major Tcp subunit [the product of the *tcpA* gene (2)] and the outer membrane protein OmpU. Expression of OmpU is regulated by ToxR in *V. cholerae* O395 (8) and, generally, when Ctx and Tcp are highly expressed, OmpU is the major outer membrane protein (8).

Typical of its altered expression of the ToxR regulon, strain 569B expressed OmpT and OmpU simultaneously (Fig. 1). Introduction of the *toxR55* mutation into 569B (strain 569B-55) had very little effect on expression of TcpA and CtxB (Fig. 1), although OmpU expression was not detectable in this mutant (Fig. 1). In contrast, introduction of the *toxR55* mutation into strain O395 (O395-55) abolished expression of CtxB, TcpA, and OmpU (Fig. 1). Transfer of the ToxR$^+$ plasmid pVM53-D (8) into O395-55 and 569B-55 complemented both *toxR* mutants, leading to identical gel profiles (Fig. 1). Thus, strain 569B is apparently less dependent on ToxR for expression of Tcp and Ctx but remains dependent on ToxR for OmpU expression. This result also suggests that strain 569B may express a regulator distinct from ToxR that is capable of activating *ctx* and *tcp* promoters.

Isolation of the *toxT* Gene from *V. cholerae* 569B. To identify activators other than ToxR that can activate the cholera toxin promoter, we repeated the original screen used to clone *toxR* (4). Briefly, plasmid DNA was isolated from a library of strain 569B and used to transform VM2, a *ctx*::*lacZ* reporter strain

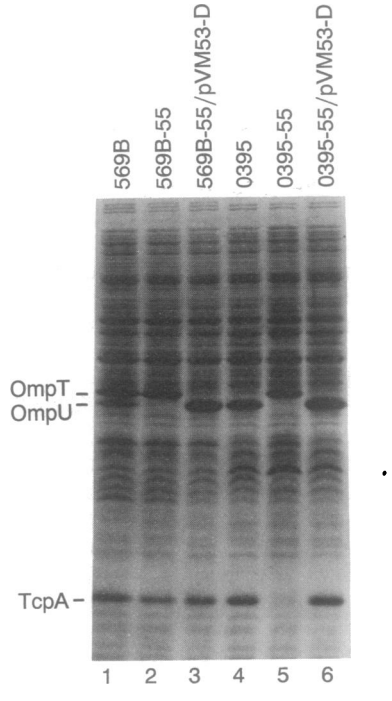

FIG. 1. SDS/PAGE profile of wild-type and *toxR* mutants of *V. cholerae* 569B and O395. Overnight cultures grown at 30°C in LB medium (pH 7.0) were resuspended in 2× SDS sample buffer (8) and boiled for 5 min. Samples of these extracts were analyzed by SDS/PAGE followed by Coomassie blue staining. The positions of the ToxR-regulated outer membrane proteins OmpT and OmpU (8) and the pilin subunit TcpA are noted. 569B-55 and O395-55 are insertion mutations in *toxR* generated by site-specific recombination as described (7, 8). 569B-55/pVM53-D and O395-55/pVM53-D are these *toxR* insertion mutants complemented with the ToxR$^+$ plasmid pVM53-D (7). Cholera toxin in the supernatants of overnight cultures was determined by GM1-ELISA (19) and reported as μg per ml per OD_{600} unit of the culture as follows. Lanes: 1, 1.8; 2, 1.4; 3, 1.9; 4, 3.5; 5, 0.01; 6, 1.8.

of *E. coli*, to ampicillin resistance. VM2 is normally white on LB agar containing the chromogenic β-galactosidase substrate 5-bromo-4-chloro-3-indolyl galactoside. Therefore, transformants were scored for elevated expression of *ctx*::*lacZ* by picking blue colonies on LB/5-bromo-4-chloro-3-indolyl galactoside plates.

When the *V. cholerae* DNA inserts of these *ctx*::*lacZ* activating plasmids were compared with *toxRS*-encoding plasmids by restriction digestion and Southern blot analysis, we discovered that several of them bore similarities to each other but were different than plasmids harboring *toxR* (data not shown). After confirming that we had not cloned a gene from *V. cholerae* that encodes an activity capable of directly cleaving 5-bromo-4-chloro-3-indolyl galactoside, we termed the gene expressed by these plasmids *toxT* and selected one ToxT$^+$ plasmid (pGJ40) for further analysis.

Activation of ToxR-Regulated Genes by *toxT*. Because ToxT, like ToxR, could activate *ctx* expression in *E. coli*, we wished to determine whether ToxT would also activate other members of the ToxR regulon.

As shown in Table 1, expression of three genes of the *tcp* cluster from strain O395, *tcpA*, *tcpC*, and *tcpI* (22), were activated in *E. coli* by the plasmid encoding ToxT but not by the plasmid encoding ToxR. Likewise, ToxT activated two other genes under ToxR control in *V. cholerae*, *tagA* (*T*oxR *a*ctivated *g*ene A; ref. 3 and C.P. and J.J.M., unpublished data) and *aldA* (the *V. cholerae* gene encoding aldehyde dehydrogenase; ref. 35). The *tagA* gene was induced 70-fold and *aldA* was induced 7-fold by ToxT but neither was directly activated by ToxR alone (Table 1). As expected, both ToxR and ToxT activated the *ctx* promoter in *E. coli* (Table 1). Thus, although there is overlap in the ToxR regulon such that ToxR and ToxT can both activate *ctx* in *E. coli*, only ToxT directly activates several other genes (*tcpA*, *tcpC*, *tcpI*, *aldA*, and *tagA*) whose expression requires ToxR in *V. cholerae*.

Complementation of a *toxR* Mutant of *V. cholerae* by Cloned *toxT*. The dependence of *tag* gene expression on ToxR in *V. cholerae* but on ToxT in *E. coli* suggests that ToxR exerts control over *tag* expression through ToxT, perhaps by controlling *toxT* expression in *V. cholerae*. If this is so, then constitutive expression of *toxT* in a *toxR* mutant of *V. cholerae* might restore expression of ToxR-regulated genes.

To test this, we took advantage of our observation that *toxT* in pGJ40 is apparently under the control of the constitutive *tet* gene promoter from the cloning vector pBR327 (data not shown; complete characterization of the *toxT* gene will be presented elsewhere). To facilitate transfer into *V. cholerae*, we constructed the mobilizable plasmid pGJ2.3, which contains pGJ40 linearized at its *Pst* I site (within the *bla* gene of pBR327) and ligated into the *Pst* I site of pLAFR2 (23), maintaining *toxT* downstream of the *tet* promoter. Plasmid pGJ2.3 was then mobilized into wild-type *V. cholerae* O395 and its ToxR$^-$ derivative JJM43.

Strains O395, O395/pGJ2.3, JJM43, and JJM43/pGJ2.3 were grown overnight at 30°C in LB medium (pH 7.0); such growth conditions favor expression of the ToxR regulon (1). As expected, strain JJM43 produced nearly undetectable levels of both Ctx and TcpA (Fig. 2). Although there was no detectable change in CtxB or TcpA expression in O395/pGJ2.3, introduction of pGJ2.3 into JJM43 restored CtxB and TcpA expression (Fig. 2) but did not fully restore OmpU production (data not shown). JJM43 can be fully complemented for CtxB, TcpA, and OmpU expression by a ToxR$^+$ plasmid (data not shown).

The experiment shown in Fig. 2 suggests that although OmpU expression shows more direct dependence on ToxR, the requirement of ToxR for *ctx* and *tcp* expression in *V. cholerae* can be eliminated by constitutive expression of a second regulatory gene, *toxT*.

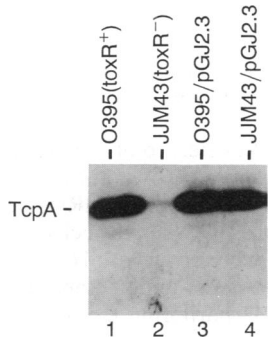

FIG. 2. Analysis of TcpA and Ctx expression by wild-type and *toxR* mutant strains of *V. cholerae* O395. Strains were grown overnight in LB medium (pH 7.0) at 30°C. Total cell proteins were analyzed on a Western blot with anti-Tcp antisera. Supernatants were used for assaying Ctx by a GM1-ELISA as described (19). Ctx in μg per ml of supernatant per OD_{600} unit of the culture is as follows. Lanes: 1, 9.1; 2, 0.02; 3, 11; 4, 7.3.

Transcription of *toxT* Depends on ToxR. Given that transcription of *toxT* from a constitutive promoter overcomes a ToxR deficiency in *V. cholerae*, the most direct explanation for the observations presented in the previous two sections is that ToxR controls the expression of *toxT*, the product of which then activates other genes under ToxR control in *V. cholerae*.

To investigate this possibility, we analyzed mRNA from O395(*toxR*$^+$) and JJM43(*toxR*$^-$). As a probe, we used an *Eco*RV restriction fragment from pGJ40 into which mapped a majority of Tn*phoA* insertions that abolish ToxT activity (V.J.D. and J.J.M., unpublished data). As shown in Fig. 3, there was abundant *toxT* mRNA in strain O395 but no detectable *toxT* mRNA in JJM43. This indicates that transcription of the *toxT* gene or the stability of the *toxT* mRNA is under the control of ToxR.

ToxR-dependent *toxT* Expression Is Modulated by Environmental Signals. Expression of the ToxR regulon in *V. cholerae* is modulated by environmental signals. For example, maximal expression of ToxR-regulated genes is observed when cells are grown in L broth at pH 6.5, whereas such expression is undetectable when cells are grown in L broth at

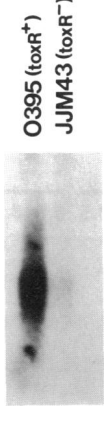

FIG. 3. Northern blot analysis of toxT-specific mRNA in wild-type and *toxR* mutant strains of *V. cholerae* O395. Strains were grown in LB medium (pH 7.0) to midlogarithmic phase at 30°C. RNA and Northern blots were prepared and probed with nick-translated restriction fragments. The probe used was a ^{32}P-labeled *Eco*RV restriction fragment isolated from the *toxT*$^+$ plasmid pGJ40. An *Eco*RI fragment derived from the *Eco*RV fragment was also used as a probe in Northern blots with the same result. This blot was stained with methylene blue (17) to ensure that equivalent amounts of RNA were loaded into each lane (data not shown).

pH 8.5 (1, 7, 8). Since the work presented above suggests that ToxR control of virulence gene expression is mediated through its control over *toxT* expression, we wished to determine whether this control exhibits environmental modulation.

Fig. 4 shows a Northern blot experiment in which RNA was isolated from strains grown at pH 6.5 or pH 8.5 and the blot was probed for *toxT*-specific mRNA. In wild-type O395, toxT mRNA was present when the cells were grown at pH 6.5 but not when they were grown at pH 8.5. In the *toxR* mutant JJM43, toxT mRNA was undetectable under either growth condition. In JJM43 harboring the ToxT$^+$ plasmid pGJ2.3, toxT mRNA was abundant in cells grown at both pH 6.5 and pH 8.5 (Fig. 4). In all cases, when toxT mRNA was present, the strain also synthesized cholera toxin (Fig. 4).

The results presented in Fig. 4 indicate that influence of the environment on the ToxR regulon may be due to modulation of *toxT* expression by ToxR. This experiment also supports the observation made above (Fig. 3) that plasmid pGJ2.3 expresses *toxT* independently of ToxR, since toxT mRNA is not detectable in strain JJM43 (*toxR*$^-$) but is present in JJM43/pGJ2.3.

DISCUSSION

The *toxR* gene of *V. cholerae* was originally identified by its ability to directly activate expression of the *ctx* promoter in *E. coli* (4). Subsequent studies have shown that *toxR* is required for expression of several other virulence genes in *V. cholerae* (2, 3), but direct control of their transcription by ToxR in a heterologous host such as *E. coli* has not been demonstrated. Identification of the *toxT* gene as described in this report may account for how ToxR activates expression of genes in *V. cholerae* that it cannot activate in *E. coli*.

As demonstrated in Table 2, five genes originally identified as being under ToxR control in *V. cholerae* are activated in *E. coli* by ToxT but not by ToxR. ToxR-regulated gene products like Tcp and Ctx are not expressed in *V. cholerae toxR* mutants (2, 3, 8). However, plasmid pGJ2.3, which constitutively expresses ToxT, can complement a *V. cholerae toxR* deletion mutant (JJM43) for expression of Tcp and Ctx (Fig. 2). Thus, although the expression of the genes in the ToxR regulon is dependent on ToxR in *V. cholerae*, these genes can be activated in the *toxR*$^-$ background by constitutively expressed ToxT. This dependence on ToxR is evidently at the level of *toxT* transcription, as indicated by the absence of detectable toxT mRNA in the *V. cholerae toxR* mutant JJM43 (Fig. 3).

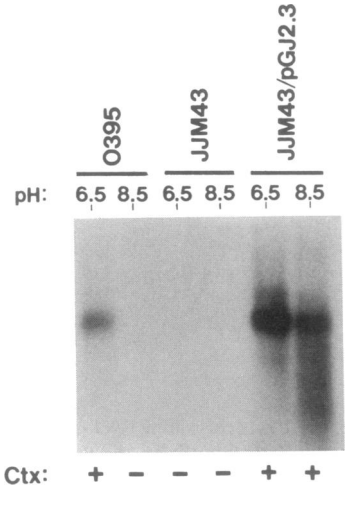

FIG. 4. Northern blot analysis of toxT mRNA in various *V. cholerae* strains in response to pH. Strains were grown in LB medium adjusted to pH 6.5 with HCl or to pH 8.5 with NaOH. Northern blots were probed with nick-translated *toxT* DNA. Cholera toxin was assayed as described (19). +, Cholera toxin present; −, cholera toxin absent.

Table 2. Activation of ToxR-regulated genes by ToxT in *E. coli*

Reporter	Induced reporter activity		
	No activator	ToxR	ToxT
ctx–phoA	215	3330 (15.5)	1490 (6.9)
tagA–phoA	8	1 (0.9)	560 (70)
tcpA–phoA	51	82 (1.6)	762 (14.9)
tcpC–phoA	14	13 (0.9)	241 (17)
tcpI–phoA	2	2 (1.0)	84 (48)
aldA–lacZ	365	230 (0.6)	2700 (7.4)

See Table 1 for plasmids encoding the reporters and activators. Reporter activities were alkaline phosphatase and β-galactosidase activities and are expressed as Miller units (15). Numbers in parentheses represent the induction ratio (activity with activator/activity without activator).

The dependence on ToxR for *toxT* expression can be alleviated by expression of *toxT* from a constitutive promoter, as in pGJ2.3 (Fig. 4). Furthermore, in wild-type *V. cholerae* grown at pH 8.5, a condition that represses the ToxR regulon, toxT mRNA is undetectable. Both *toxT* expression and *ctx* expression were detected at pH 8.5 in the absence of ToxR when *toxT* was under the control of another promoter (Fig. 4). This indicates that environmental modulation is controlled by ToxR-activated expression of *toxT*.

The data presented in this report are thus consistent with a model of regulatory cascading in which ToxR directs coordinate control of virulence gene expression in *V. cholerae* by activating expression of *toxT*. The *toxT* gene product then directly activates expression of several ToxR-controlled genes. To date, we do not know whether ToxR directly or indirectly controls *toxT* expression, although preliminary evidence favors the former possibility (V.J.D. and J.J.M., unpublished observations). Also, the nature of the *toxT* gene product remains to be determined. Since ToxT and ToxR can activate the same promoter (*ctx*), we expect the *toxT* gene product to show, as does ToxR (7), some similarity to a known class of transcriptional activators or perhaps even an RNA polymerase σ factor.

The discovery of ToxT stemmed in part from the observation that a *toxR* insertion mutant of strain 569B still expressed several genes belonging to ToxR regulon (Fig. 1). This strain synthesizes cholera toxin under growth conditions that are not permissive for toxin expression by most other strains (1, 24). In addition, strain 569B carries a deletion of the *toxS* gene (5). Deletion of or insertion into the *toxS* gene of strain O395 reduces expression of the ToxR regulon and decreases the ability of this strain to colonize infant mice (25). It therefore appears that strain 569B may have largely dispensed with the requirement for ToxR to express virulence genes. If our model is accurate, one way that this might have occurred is if the strain has an altered *toxT* promoter, thereby rendering it largely independent of ToxR for its expression. Supporting this hypothesis is our observation that strain JJM43/pGJ2.3, which does not express *toxR* but expresses *toxT* constitutively, has essentially the same constitutive phenotype as does strain 569B. Characterization of the *toxT* promoter from 569B and more typical strains like O395 should allow testing of this hypothesis.

Understanding the molecular mechanisms controlling the regulation of virulence determinants in response to environmental signals in pathogenic microorganisms is currently of great interest. Control of virulence gene expression by members of the two-component regulatory family of activators (26) has been demonstrated in many species (1, 27, 28), but there are only a few examples in which regulators have been

shown to directly activate the promoters they control in a heterologous organism like *E. coli* (29–31). For example, in *Bordetella pertussis* several genes that encode virulence determinants are under the coordinate control of the products of the *bvg* locus, yet apparently only one of these genes (*fha*) is directly activated by Bvg (31). Likewise, in *Pseudomonas aeruginosa*, the *regA* gene product is required for expression of the *toxA* gene encoding exotoxin A (32), but RegA does not directly bind to or activate the *toxA* promoter (33). It is likely that a regulatory cascade operates in these and other systems as well.

The ToxR regulatory cascade may expand beyond the *toxT* gene. For example, recent evidence suggests that the expression of the *toxR* gene is modulated by the heat-shock response and controlled by the level of σ-32 in the cell (34). Furthermore, one of the genes shown to be activated by ToxT in this report is the *tcpI* gene, which itself appears to be a regulator of Tcp expression (22). If ToxT and other possible "downstream regulators" such as TcpI are themselves responsive to specific environmental conditions, then this system of cascading regulatory proteins would be a powerful mechanism for *V. cholerae* to fine tune expression of virulence determinants throughout its pathogenic cycle. Such a regulatory strategy might help virulent microorganisms deal with the rapid changes in environment that occur as a consequence of the pathophysiology of infection. Cascading may also be important to confer orderly temporal control over gene expression in much the same way as has been observed in developmental cycles such as sporulation (9).

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