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Differential Transcription of the *tcpPH* Operon Confers Biotype-Specific Control of the *Vibrio cholerae* ToxR Virulence Regulon

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Epidemic strains of *Vibrio cholerae* O1 are divided into two biotypes, classical and El Tor. In both biotypes, regulation of virulence gene expression depends on a cascade in which ToxR activates expression of ToxT, and ToxT activates expression of cholera toxin and other virulence genes. In the classical biotype, maximal expression of this ToxR regulon in vitro occurs at 30°C at pH 6.5 (ToxR-inducing conditions), whereas in the El Tor biotype, production of these virulence genes only occurs under very limited conditions and not in response to temperature and pH; this difference between biotypes is mediated at the level of *toxT* transcription. In the classical biotype, two other proteins, TcpP and TcpH, are needed for maximal *toxT* transcription. Transcription of *tcpPH* in the classical biotype is regulated by pH and temperature independently of ToxR or ToxT, suggesting that TcpP and TcpH couple environmental signals to transcription of *toxT*. In this study, we show a near absence of *tcpPH* message in the El Tor biotype under ToxR-inducing conditions of temperature and pH. However, once expressed, El Tor TcpP and TcpH appear to be as effective as classical TcpP and TcpH in activating *toxT* transcription. These results suggest that differences in regulation of virulence gene expression between the biotypes of *V. cholerae* primarily result from differences in expression of *tcpPH* message in response to environmental signals. We present an updated model for control of the ToxR virulence regulon in *V. cholerae*.

Vibrio cholerae, a curved gram-negative bacterium, causes a secretory diarrhea in humans that can produce severe dehydration and death (23). Strains of *V. cholerae* that cause epidemic cholera belong to serogroup O1. *V. cholerae* O1 is divided into two biotypes, classical and El Tor, which differ biochemically and phenotypically in such properties as susceptibility to polymyxin B, ability to hemolyze sheep erythrocytes, and the Voges-Proskauer reaction. Human infections with these two biotypes also differ clinically; infection with classical *V. cholerae* O1 more frequently produces severe infection than does El Tor, suggesting that the genetic and phenotypic differences between the two biotypes may also be reflected in their pathogenic potential. In 1992, a new serogroup of *V. cholerae* emerged as a cause of epidemic cholera in southeast Asia, *V. cholerae* O139 (40). Several lines of evidence suggest that this new serogroup is closely related to and derived from the El Tor biotype of *V. cholerae* O1 by substitution of genes encoding the O139 O antigen and acquisition of the ability to produce a capsule (1, 4, 7, 8, 48, 50).

The major virulence factor for all of these pathogenic strains of *V. cholerae* is cholera toxin, a heterodimeric protein exotoxin that consists of a single, enzymatically active A subunit noncovalently associated with five B subunits (13). The pentamer of B subunits binds the holotoxin to its receptor, the ganglioside GM_1, on intestinal epithelial cells. Upon binding, the A subunit is nicked, reduced, and translocated intracellularly, where it acts as an ADP-ribosyl transferase, elevating cyclic AMP levels within the cell and causing a secretory diarrhea (6, 14, 15, 30). The genes for the A and B subunits of cholera toxin are contained in an operon (31) within a filamentous bacteriophage that lysogenizes *V. cholerae*, the CTX phage (51). In classical strains of *V. cholerae*, expression of cholera toxin in vitro is strongly regulated by environmental growth conditions, such as pH, temperature, and osmolarity (35, 36). How these in vitro growth conditions relate to the in vivo expression of cholera toxin is not yet certain.

A second major virulence factor of *V. cholerae* is the toxin-coregulated pilus (TCP), a colonization factor whose expression is regulated in classical strains by the same environmental growth conditions as those involved in the expression of cholera toxin (45). The major subunit of TCP is encoded by *tcpA*; several other genes involved in the biosynthesis, processing, and assembly of TcpA into the mature pilin structure are located downstream of *tcpA* on the *V. cholerae* chromosome (46). Recently, these and adjacent genes were found to be located on a *V. cholerae* pathogenicity island within another filamentous bacteriophage, designated VPIϕ (24, 25).

Expression of cholera toxin and TCP is dependent on a regulatory gene in *V. cholerae*, *toxR*, which encodes a transmembrane protein with an amino-terminal, cytoplasmic DNA binding domain that acts as a transcriptional activator (34, 36). A second regulatory gene, *toxS*, is transcribed in an operon with *toxR* and encodes a periplasmic protein that facilitates dimerization and activation of ToxR as a DNA-binding protein (10, 33). ToxR is essential for activation of cholera toxin and TCP expression in *V. cholerae*. Although ToxR is sufficient to

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activate transcription of the *ctxAB* promoter in *E. coli*, it is not able to activate transcription from either the *ctxAB* or *tcpA* promoter directly in a *V. cholerae* background (2, 34). Instead, ToxR activates transcription of an additional regulatory gene, *toxT*, that encodes a transcriptional activator in the AraC family (12, 21, 37). ToxT then directly activates transcription at the *tcpA* and *ctxAB* promoters, as well as other genes that are part of the ToxR regulon. This suggests a regulatory cascade for expression of cholera virulence factors in which ToxR activates the gene coding for expression of ToxT, and ToxT in turn activates the genes coding for expression of cholera toxin and TCP, as well as other genes in the ToxR regulon (9).

The gene encoding ToxT is located in the *tcp* gene cluster downstream of *tcpA*, between *tcpF* and *tcpJ* (21, 26, 39). Transcription of *toxT* occurs both as part of the *tcpA* operon, as well as separately from a ToxR-dependent promoter located between *tcpF* and *toxT* (2, 20). There are several inverted repeats upstream of this *toxT* promoter in classical *V. cholerae*, and these repeats have been shown to be necessary for binding of ToxR and activation of *toxT* transcription. However, ToxR is not sufficient in *Escherichia coli* to activate transcription of an operon fusion between the classical *toxT* promoter and *lacZ* and indeed represses transcription at the *toxT* promoter (20). This suggests the possibility that an additional protein or proteins are necessary in classical *V. cholerae* for full activation at the *toxT* promoter.

Previous investigators have identified additional genes upstream of *tcpA*, including *tcpP* and *tcpH*, which are transcribed as an operon in the same direction as *tcpA*, and *tcpI*, which is transcribed divergently from *tcpPH* (38, 46). We and Häse and Mekalanos have previously shown that transcription of *toxT* in classical *V. cholerae* requires the gene products encoded by *tcpPH* (5, 18). While TcpP alone appears to be sufficient for *toxT* expression, activation is greatly enhanced when TcpH is coexpressed (18). The proposed membrane topology suggests that TcpP and TcpH may be localized to the cytoplasmic membrane, similar to ToxR and ToxS (18). Other studies indicate that classical TcpP and TcpH act in concert with ToxR and ToxS to activate *toxT* transcription, although the exact mechanism is not yet understood (27, 28).

Whereas expression of ToxR in classical *V. cholerae* is constitutive, transcription of *toxT* is regulated by the same environmental growth conditions that regulate expression of cholera toxin and TCP (9, 11, 21). We have previously shown that transcription of *tcpPH* in classical *V. cholerae* is regulated by pH and temperature, suggesting that the protein products, TcpP and TcpH, may couple environmental growth conditions to transcription of *toxT* and expression of the ToxR regulon in classical *V. cholerae* (5).

Expression of cholera toxin and TCP in El Tor strains of *V. cholerae* is regulated quite differently in response to environmental conditions from expression in classical strains of *V. cholerae*. In the El Tor biotype, production of cholera toxin and TCP occurs only under very limited in vitro growth conditions, referred to as AKI conditions (49), but not under the conditions of temperature and pH that induce expression of the ToxR regulon in classical *V. cholerae*. DiRita et al. (11) have previously shown that these differences between *V. cholerae* biotypes in expression of the ToxR regulon in response to environmental conditions are mediated at the level of *toxT* transcription and that overexpression of *toxT* from a heterologous promoter in an El Tor strain leads to expression of cholera toxin and TCP without the requirement for AKI conditions. We wished to examine whether the difference in transcription of *toxT* in response to environmental conditions between the two biotypes of *V. cholerae* reflects differences in expression of *tcpPH* message and/or the activity of the TcpP and TcpH proteins.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains and plasmids used in this study are shown in Table 1. Plasmid pUC19 was used as the vector for cloning PCR products, and pLAFR3 was used for complementation experiments. Plasmids were initially cloned in *E. coli* DH5α. After characterization, recombinant plasmids were electroporated into *V. cholerae* by using a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) according to the manufacturer's protocol, modified for electroporation into *V. cholerae* as previously described (16). The electroporation conditions were 2,500 V at 25-μF capacitance, producing time constants of 4.5 to 4.8 ms. To construct a polar mutation in the *V. cholerae tcpP* locus, a 230-bp internal fragment from *tcpP* was cloned into the suicide plasmid pGP704, resulting in plasmid pYM2-16. *E. coli* SM10λ*pir* was used to mobilize plasmid pYM2-16 into *V. cholerae* O395 by conjugation, resulting in YM2-21. Proper integration of the suicide plasmid within *tcpP* was confirmed by Southern blotting. *V. cholerae* KSK401 was isolated following Tn*phoA* mutagenesis of strain KSK218, by using a screen for reduced activity of the *ctx*::*lacZ* reporter activity in KSK218 (42). The Tn*phoA* insertion in KSK401 was cloned by chromosomal capture and backcrossed to confirm that the phenotype was linked to the insertion; DNA sequencing was used to determine that Tn*phoA* had inserted within *tcpP*. In order to obtain an *E. coli* reporter strain that could be used with clones of *tcpPH* in pLAFR3 (Tc^r), bacteriophage P1 transduction was utilized to move the *toxT*::*lacZ* fusion, which is linked to kanamycin resistance in DH92, into *E. coli* MC4100 (Tc^s *recA*$^+$ *lacZ*), resulting in strain RT4146. Strains were stored in 15% glycerol at −70°C and inoculated onto Luria-Bertani (LB) agar medium prior to growth in LB liquid culture. (LB medium contained 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter.) LB liquid cultures were grown on a tube roller at 30°C at pH 6.5 to induce ToxR-dependent gene expression and at 37°C at pH 8.4 to repress ToxR-dependent gene expression (35). Overnight cultures grown under ToxR-inducing conditions with moderate aeration were examined for autoagglutination as described previously (45); autoagglutination reflects expression of TCP. AKI conditions were as described previously (49). Ampicillin (25 or 100 μg/ml), streptomycin (100 μg/ml), kanamycin (45 μg/ml), tetracycline (5 or 15 μg/ml), or chloramphenicol (34 μg/ml) was added as appropriate.

Genetic methods. Isolation of plasmid and bacterial chromosomal DNA, restriction enzyme digests, agarose gel electrophoresis, and Southern hybridization analysis were performed according to standard molecular biologic techniques (41). DNA restriction endonucleases, T4 DNA ligase, T4 DNA polynucleotide kinase, Klenow fragment, and shrimp alkaline phosphatase were used according to the manufacturers' specifications. DNA fragments used as probes for Southern and Northern hybridizations were generated by PCR as previously described (5), separated on 1% agarose gels, cut out from the gel under UV illumination, and recovered with a Compass kit (American Bioanalytical, Natick, Mass.). Purified DNA fragments were radiolabelled with [α-^{32}P]dCTP (Dupont Biotechnology Systems, NEN Research Products, Boston, Mass.) by using a random-priming labelling kit (Prime-It II; Stratagene, La Jolla, Calif.). GeneScreen and GeneScreen Plus hybridization transfer membranes (DuPont Biotechnology Systems) were used according to the manufacturer's protocols for Northern and Southern hybridizations, respectively. DNA sequencing was performed at the Massachusetts General Hospital, Department of Molecular Biology, DNA Sequencing Core Facility, by using ABI Prism DiTerminator Cycle sequencing with AmpliTaq DNA polymerase FS and an AB1377 DNA sequencer (Perkin-Elmer Applied Biosystems Division, Foster City, Calif.).

Bacterial RNA was isolated by using CATRIMOX-14 (Qiagen, Valencia, Calif.). Five milliliters of mid-log-phase bacterial cultures grown under appropriate growth conditions was quickly added to 5 ml of crushed ice solution A (75 ml of TM buffer [see below], 1.7 ml of 1 M sodium azide) and centrifuged at 4°C. The bacterial pellet was suspended in 400 μl of TM buffer (10 mM Tris HCl [pH 7.2], 5 mM $MgCl_2$), 4 μl of DNase I (RNase-free; Boehringer-Mannheim, Indianapolis, Ind.), and 100 μl of lysozyme (4-mg/ml final concentration). The suspension was frozen in a dry ice-ethanol bath and thawed at 37°C four times. After the fourth thaw, the suspension was frozen again, and 67 μl of 20 mM acetic acid was added to the frozen cells. The cells were then thawed at 37°C, and 500 μl of CATRIMOX-14 was added immediately upon thawing. The mixture was centrifuged for 1 min at 6,500 rpm in an Eppendorf microcentrifuge. The pellet was resuspended in 1 ml of 2 M $LiCl_2$ and incubated at room temperature for 5 min. The suspension was then spun at top speed in a microcentrifuge for 6 min at 4°C. The pellet was again resuspended in 1 ml of $LiCl_2$, incubated, and centrifuged as described above. The pellet was air dried and resuspended in 100 μl of RNase-free water overnight at 4°C before the RNA concentration was measured. Ten micrograms of purified RNA was denatured with glyoxal for Northern blot analysis as previously described (3).

Assays. Cholera toxin production was assayed in culture supernatants with a GM_1 enzyme-linked immunosorbent assay as described previously (22). β-Galactosidose activity was determined as described previously (32). Induction of plasmid-encoded *V. cholerae tcpPH* under the control of the *lacZ* promoter in *E. coli* was achieved with 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma,

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
V. cholerae		
O395	Classical strain; Sm^r	Laboratory collection
O395 *toxR*	O395 with mutation in *toxR* created by integration of suicide plasmid pVM55; Sm^r Ap^r	52
C6709	El Tor strain; Sm^r	Laboratory collection
KTT7-1	O395 *tcpH*; Sm^r	5
KSK218	O395 Δ*lacZ ctx*::*lacZ*	42
KSK401	KSK218 *tcpP*::Tn*phoA* (polar on *tcpH*)	This study
YM2-21	O395 with a polar mutation in *tcpP* created by integration of the suicide plasmid pYM2-16; Sm^r Ap^r	This study
E. coli		
DH5α	F^- *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF-lacZYA*)*U169* (Φ80d *lacZ*ΔM15)	17
SM10λpir	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir R6K*	35
DH92	TE2680[Km^r-p*toxT*::*lacZ*]	20
RT4146	MC4100[Km^r-p*toxT*::*lacZ*], by P1 transduction from DH92	This study
CC118	Δ(*ara leu*)*7697* Δ(*lac*)*X74 araD139 phoA20 galE galK thi rpsE rpoB argE recA1*	29
Plasmids		
pLAFR3	Cloning vector; Tc^r	44
pACYC184	Cloning vector; Cm^r Tc^r	Laboratory collection
pUC19	Cloning vector; Ap^r	Gibco-BRL
pGP704	Suicide vector; Ap^r	35
pPAC22	PCR product of the wild-type *tcpPH* genes from *V. cholerae* O395, with *Bam*HI ends, cloned into pUC19 for sequencing and subsequent constructions; Ap^r	5
pYM49	*Bam*HI fragment from pPAC22, cloned into pLAFR3 in same orientation to the *lacZ* promoter; Tc^r	This study
pYM53	PCR product of *tcpPH* genes from *V. cholerae* C6709, cloned into pUC19 for sequencing and subsequent constructions; Ap^r	This study
pYM59	*Bam*HI fragment from pYM53 containing *tcpPH* genes from C6709, cloned into pLAFR3 in same orientation to *lacZ* promoter; Tc^r	This study
pYM2-16	230-bp *tcpP* PCR product from *V. cholerae* O395 cloned into pGP704; Ap^r	This study
pTSK	pACYC184[p*tet*::*toxRS*]; Cm^r	2
pTSS-5	pACYC184[p*tet*::*toxT*]; Cm^r	2
pRAB	pRZ5202 [*tcpAB*::*lacZ*]; Ap^r	2
pRX	pRZ5202[*ctx*::*lacZ*]; Ap^r	2
pVM55	Suicide plasmid pJM703.1, containing a 630-bp *Eco*RI-*Hpa*I internal fragment of *toxR*; Ap^r	35

[a] Sm, streptomycin; Ap, ampicillin; Km, kanamycin; Tc, tetracycline; Cm, chloramphenicol.

St. Louis, Mo.); since *V. cholerae* does not contain *lacI*, the same plasmid constructs gave constitutive expression of *tcpPH* in *V. cholerae*.

RESULTS

Transcription of *tcpPH* in response to pH and temperature differs between classical and El Tor strains of *V. cholerae*. We have previously shown that expression of *tcpPH* message is regulated by temperature and pH, independently of ToxR or ToxT, in classical *V. cholerae* (5). To determine if expression of *tcpPH* message could account for the differential expression of virulence factors between the two biotypes, Northern blot hybridization was utilized. When grown under ToxR-inducing conditions, classical strain O395 produced substantial amounts of *tcpPH* message, while El Tor strain C6709 produced significantly less (Fig. 1). Both strains produced similar levels of transcript when grown under AKI conditions (data not shown), although the levels were reduced compared to that of O395 grown under ToxR-inducing conditions. Neither strain produced significant amounts of *tcpPH* transcript when grown under ToxR-repressing conditions. The *tcpPH* mutant strain, YM2-21, did not produce any message when grown under either ToxR-inducing or ToxR-repressing conditions. Cholera toxin production mirrored *tcpPH* expression. Transcripts of *tcpA* and *toxT* message were absent from C6709 grown under ToxR-inducing conditions, the latter confirming observations reported by DiRita et al. (11). Neither O395 nor C6709 transcribed *toxT* nor *tcpA* under ToxR-repressing conditions.

Plasmids expressing either classical or El Tor TcpP and TcpH can complement a classical *tcpPH* mutant. To determine if plasmid clones of *tcpP* and *tcpH* from each biotype were functional, we examined the ability of these constructs to complement a classical *V. cholerae tcpPH* mutant. The classical *V. cholerae* strain KSK218 contains a *ctx*::*lacZ* operon fusion in both chromosomal copies of the cholera toxin operon in classical strain O395. Inactivation of *tcpP* and *tcpH*, by Tn*phoA* insertion within *tcpP* to produce strain KSK401, reduced expression at the *ctx* promoter, as reflected by β-galactosidase activities (Table 2). The TcpP and TcpH proteins expressed from pYM49 (classical) and pYM59 (El Tor) were each able to restore *ctxAB* transcription, as well as autoagglutination (a rough measure of TCP expression), in KSK401. This confirms that both clones are functional and suggests that there is little difference between the biotypes with respect to the activity of the TcpP and TcpH proteins when they are present in the classical biotype background.

Differences in environmental regulation of the ToxR regulon between classical and El Tor *V. cholerae* are overcome by overexpressing TcpP and TcpH. Although classical strain O395

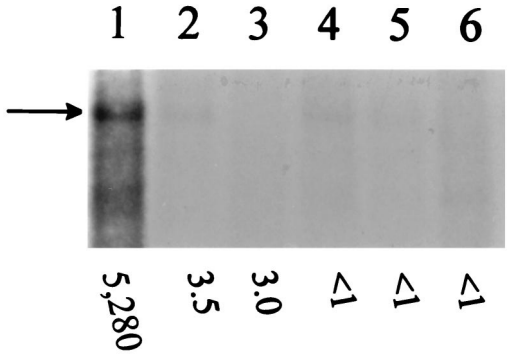

FIG. 1. Northern blot analysis of 10 μg of total RNA from O395 (lanes 1 and 4), C6709 (lanes 2 and 5), and YM2-21 (lanes 3 and 6) grown under ToxR-inducing (lanes 1 to 3) and ToxR-repressing (lanes 4 to 6) conditions. A 458-bp fragment containing *tcpPH* was used as a probe. Arrow indicates 1.28 kbp of message. The values at the bottom indicate corresponding cholera toxin measurements (nanograms per milliliter at OD_{600}).

showed a more than 4,000-fold increase in expression of cholera toxin in response to ToxR-inducing conditions, El Tor strain C6709 showed no increase (Table 3); however, both strains were able to express comparable amounts of cholera toxin in response to AKI conditions (not shown). Overexpression of TcpP and TcpH in the El Tor strain resulted in a marked increase in cholera toxin expression; plasmid pYM59, which overexpresses the El Tor *tcpP* and *tcpH* genes from the *lacZ* promoter, stimulated strain C6709 to produce levels of cholera toxin under ToxR-inducing conditions comparable to those of wild-type O395. Overexpression of *tcpPH* from this constitutive promoter resulted in expression of cholera toxin even under ToxR-repressing conditions. However, a small amount of residual regulation of expression in response to temperature and pH was still observed even when the *tcpPH* genes were expressed from the *lacZ* promoter.

TcpP and TcpH from both the classical and El Tor biotypes work synergistically with ToxR and ToxS to activate *toxT* transcription in *E. coli*. To better understand the interaction of TcpP and TcpH with the ToxR regulon, we examined the ability of these proteins from either biotype to activate the *ctx*, *tcpA*, and *toxT* promoters independently of other *V. cholerae* proteins. Plasmids containing the appropriate regulatory genes, under the control of constitutive or inducible promoters, were tested for their ability to activate classical *V. cholerae* promoter fusions in *E. coli* (Table 4). We used a chromosomal *toxT*::*lacZ* fusion, because a plasmid-encoded fusion produced levels of β-galactosidase too high for accurate measurement. As shown previously (12), ToxRS and ToxT both activated the *ctxAB* promoter, while only ToxT activated the *tcpA* promoter; ToxRS repressed the *toxT* promoter approximately twofold. TcpPH from both biotypes had no effect on transcription from the *ctxAB* or *tcpA* promoters, but both activated transcription of *toxT* approximately twofold. The combination of ToxRS and TcpPH (from either biotype) activated transcription from the *toxT* promoter approximately 10-fold. This had previously been suggested to be the case for classical *V. cholerae* in preliminary data by Krukonis et al. (27).

TABLE 2. Measurements of β-galactosidase activity and autoagglutination for a classical biotype *ctx*::*lacZ V. cholerae* strain grown under ToxR-inducing conditions

Strain	β-Galactosidase activity (Miller units/OD_{600})[a]	Autoagglutination
KSK218	1,750 (1,635–1,943)	+
KSK401	97 (95–98)	−
KSK401(pLAFR3)	143 (135–151)	−
KSK401(pYM49)	4,848 (4,406–5,433)	+
KSK401(pYM59)	3,412 (3,259–3,670)	+

[a] The mean and range (in parentheses) of at least three independent experiments are shown.

TABLE 3. Measurements of cholera toxin in culture supernatants following overnight growth under the indicated conditions

Strain	Amt of cholera toxin (ng/ml/OD_{600}) under[a]:	
	ToxR-inducing conditions	ToxR-repressing conditions
O395	4,464 (2,966–6,041)	<1
C6709	<1	<1
C6709(pLAFR3)	<1	<1
C6709(pYM59)	4,030 (2,512–6,478)	794 (493–1,041)

[a] The mean and range (in parentheses) of at least three independent experiments are shown.

DISCUSSION

Production of cholera toxin and TCP is regulated differently between the two biotypes of *V. cholerae* O1. Classical strains of *V. cholerae* express these virulence factors under both ToxR-inducing conditions and AKI conditions, whereas El Tor strains of *V. cholerae* O1 express detectable levels only under AKI conditions in vitro. ToxR is required in both biotypes for expression of these virulence factors, although the expression of ToxR itself is independent of environmental growth conditions (9, 11). ToxT is also required for expression of these virulence factors in both biotypes, and expression of *toxT* in response to environmental conditions differs between the two biotypes (11). ToxT is expressed in classical strains of *V. cholerae* under both ToxR-inducing and AKI conditions, whereas El Tor strains of *V. cholerae* express ToxT only under AKI conditions. Expression of ToxT from an inducible promoter in an El Tor strain of *V. cholerae* allows this biotype to make cholera toxin and TCP without the requirement for AKI conditions (11). This suggests that the biotype-specific expression of cholera virulence factors is the result of differential expression of *toxT* in response to environmental growth conditions. DiRita et al. (11) suggested that another factor may be involved in activating *toxT* in *V. cholerae* and that this factor may be active only under AKI conditions in El Tor strains, but in a broader range of growth conditions in classical strains of *V. cholerae*. They also suggested that the resultant differential control of toxin production between the two biotypes may contribute to differences in their pathogenicity.

We have previously shown that transcription of *toxT* in classical *V. cholerae* requires TcpH (5). Transcription of the *tcpPH* operon is regulated by pH and temperature in classical strains, suggesting that TcpP and TcpH may couple the environmental signals of temperature and pH to transcription of *toxT* and expression of the ToxR regulon in classical *V. cholerae*. In classical *V. cholerae*, both TcpP and TcpH, in coordination with ToxR and ToxS, are required for optimal transcription of the *toxT* promoter (18, 27, 28). While the role of TcpP and TcpH in activation of *toxT* transcription has been established in classical *V. cholerae*, their role in the regulation of virulence gene expression in El Tor strains of *V. cholerae* had not been explored. Thomas et al. (47) and Ogierman et al. (38) have

compared sequences of the *tcp* gene clusters of classical and El Tor *V. cholerae* and found that the major differences upstream of *tcpA* fall in the intergenic regions between *tcpI* and *tcpP* and between *tcpH* and *tcpA*, suggesting that differences in expression of *tcpPH* and/or *tcpA* message may underlie the differences in expression of cholera virulence factors between the two biotypes.

The data in the present study demonstrate that expression of *tcpPH* between the two biotypes of *V. cholerae* is fundamentally different in response to environmental signals of temperature and pH. In the classical strain, expression of *tcpPH* message is strongly induced by conditions of temperature and pH known to induce expression of the ToxR regulon, while similar conditions do not result in substantial expression of *tcpPH* message in the El Tor strain. *toxT* expression and *tcpA* expression were similarly divergent between the two biotypes, confirming previously reported results (11). These data suggest that a fundamental difference between the biotypes lies in the environmental regulation of *tcpPH* expression. Furthermore, given the role of TcpP and TcpH in expression of *toxT* in classical *V. cholerae* (5, 18), the current findings likely explain the previously reported biotype-specific expression of *toxT* (11).

TcpP and TcpH from both the classical and El Tor biotypes were able to activate expression of the ToxR regulon in several host backgrounds. The functionality of the El Tor TcpP and TcpH was established by demonstrating the ability of plasmid pYM59, encoding the El Tor TcpP and TcpH, to restore activation of a *ctx*::*lacZ* fusion in a *tcpPH* mutant of classical *V. cholerae*, strain KSK401. Levels of β-galactosidase were comparable to those seen with expression of classical TcpP and TcpH from plasmid pYM49. Similarly, overexpression of the El Tor *tcpPH* gene products in the El Tor *V. cholerae* background resulted in increased cholera toxin production, comparable to that of the classical biotype. Constitutive expression of the *tcpPH* gene products from the *lacZ* promoter in the El Tor background resulted in reduced regulation of cholera toxin expression in response to environmental conditions; however, some residual regulation remained. This may be the result of combined expression of TcpPH from both the *tcpPH* and *lacZ* promoters, but it may also suggest the involvement of an additional factor or factors in environmental regulation.

In *E. coli*, plasmids encoding either the classical or El Tor TcpP and TcpH under the control of an inducible promoter were able to activate a classical *V. cholerae toxT*::*lacZ* fusion. In both cases, the activity was greatly enhanced by the presence of ToxR and ToxS; thus, El Tor TcpP and TcpH appear to act in concert with ToxR and ToxS to amplify *toxT* expression, as was previously suggested for classical *V. cholerae* (27).

Although there are a small number of amino acid differences between the El Tor and classical TcpP and TcpH proteins, the fact that the El Tor proteins were able to activate transcription of *toxT* and expression of the ToxR regulon in either biotype and in *E. coli* indicates little difference in the functionality of these proteins compared to that of the classical TcpP and TcpH proteins. Recently, Häse and Mekalanos showed that the activity of classical TcpP and TcpH is influenced by Na^+ concentration or osmolarity (19). While our results indicate that the effects of pH and temperature on TcpP and TcpH activity do not differ between the two biotypes, we did not examine whether the effects of Na^+ concentration or osmolarity on TcpPH activity differ between the two biotypes.

The near absence of *tcpPH* message in El Tor *V. cholerae* strains when grown under ToxR-inducing conditions suggests (i) that there are differences in the *tcpPH* promoters between the two biotypes that result in altered environmental regulation, (ii) that a *trans*-acting factor(s) that is present in classical

TABLE 4. Ability of various *V. cholerae* regulatory proteins to activate the *ctx*, *tcpA*, and *toxT* promoters fused to β-galactosidase in *E. coli*

Reporter fusion	β-Galactosidase activity (Miller units/OD_{600}) with regulatory protein expression[a]						
	pACYC184 (control)	pLAFR3 (control)	pTSK (ToxRS)	pTSS-5 (ToxT)	pYM49 (classical TcpPH)	pYM59 (El Tor TcpPH)	pTSK + pYM49	pTSK + pYM59
CC118(pRX) (*ctx-lacZ*)	5 (4–6)	5 (5)	380 (360–391)	81 (70–98)	4 (4)	5 (4–5)	382 (373–389)	380 (366–397)
CC118(pRAB) (*tcpAB-lacZ*)	7 (6–8)	6 (6–7)	5 (5–6)	342 (203–518)	6 (6)	6 (6)	7 (7–8)	7 (7)
RT4146 (*toxT-lacZ*)	327 (314–345)	330 (322–337)	116 (108–128)	247 (242–254)	565 (553–574)	692 (661–726)	2,935 (2,839–3,071)	3,554 (3,476–3,687)

[a] Means and ranges (in parentheses) of triplicate measurements of β-galactosidase activities are shown. Expression of TcpPH from plasmids pYM49 and pYM59 was induced with IPTG.

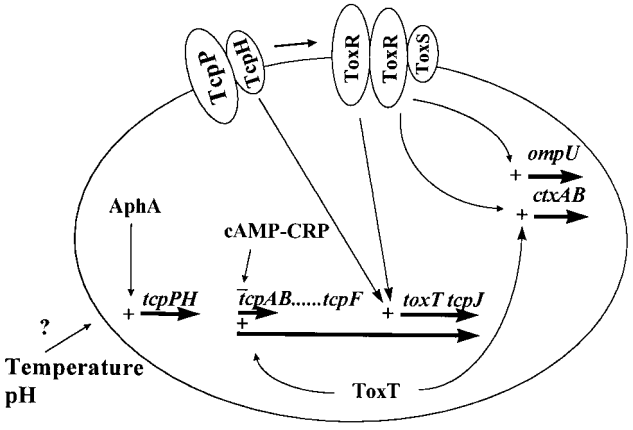

FIG. 2. Model for the regulation of the ToxR regulon in classical *V. cholerae*, incorporating previous observations as well as those in the present study (see text for discussion). +, transcriptional activation; −, repression. Transcription is represented by thick solid arrows. Additional factors regulating *tcpPH* transcription (indicated by ?) are currently under investigation. cAMP-CRP, cyclic AMP (cAMP)-cyclic AMP receptor protein.

strains but absent or altered in El Tor strains is the cause of the observed differential expression in response to temperature and pH, or (iii) that there are differences in *tcpPH* transcript stability between the two biotypes. Experiments are currently under way to investigate these hypotheses.

Recently, a protein designated AphA was found to be required for activation of *tcpPH* expression in classical *V. cholerae*, although the mechanism of activation is not known (43). Expression of *aphA* does not appear to be influenced by environmental conditions that activate *tcpPH* expression in classical *V. cholerae*. It is not yet known whether AphA plays a role in the differential expression of *tcpPH* in the classical and El Tor biotypes.

The results presented here, in addition to previously reported results (5, 18, 27), are consistent with a model in which TcpP and TcpH, in conjunction with ToxR and ToxS, activate transcription of *toxT* in classical and El Tor strains of *V. cholerae*, with subsequent expression of the ToxR regulon. An updated model for regulation of virulence gene expression in classical *V. cholerae* is depicted in Fig. 2.

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