

## Classical and El Tor Biotypes of *Vibrio cholerae* Differ in Timing of Transcription of *tcpPH* during Growth in Inducing Conditions

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**Two protein pairs in *Vibrio cholerae*, ToxRS and TcpPH, are necessary for transcription from the *toxT* promoter and subsequent expression of cholera virulence genes. We have previously shown that transcription of *tcpPH* in classical strains of *V. cholerae* is activated at mid-log-phase growth in ToxR-inducing conditions, while transcription of *tcpPH* in El Tor strains is not. In this study, we showed that while transcription of *tcpPH* differs at mid-log-phase growth in ToxR-inducing conditions between the biotypes, transcription is equivalently high during growth in AKI conditions. We used *tcpPH::gusA* transcriptional fusions to quantitate expression of *tcpPH* in each biotype throughout growth in ToxR-inducing conditions and showed that although transcription of *tcpPH* is reduced at mid-log-phase growth in an El Tor strain, transcription is turned on later in growth to levels in excess of those in the classical strain (although cholera toxin is not produced). This suggests that the difference in expression of cholera virulence factors in response to ToxR-inducing conditions between the El Tor and classical biotypes of *V. cholerae* may be related to the timing of transcription of *tcpPH* rather than the absolute levels of transcription.**

*Vibrio cholerae* is a gram-negative bacterium that causes the watery-diarrheal illness cholera. Strains of *V. cholerae* are classified serotypically based on their O antigen, and *V. cholerae* O1 is the predominant cause of epidemic cholera. *V. cholerae* O1 is divided into two biotypes, classical and El Tor, which are distinguished by a variety of phenotypic markers (13). The major virulence factors for *V. cholerae* are cholera toxin and the toxin-coregulated pilus (TCP). Cholera toxin is a heterodimeric protein exotoxin which consists of an enzymatically active A subunit, which acts as an ADP-ribosyl transferase and elevates intracellular cyclic AMP levels, and a pentamer of B subunits, which bind holotoxin to its receptor, the ganglioside GM<sub>1</sub>, on eukaryotic cells. The genes for cholera toxin are encoded as an operon, *ctxAB*, and are contained within the genome of a filamentous phage, CTX $\phi$  (16, 29). The major subunit of the TCP is encoded by *tcpA* (26). This gene is transcribed with a larger operon of 12 genes which are involved in processing and assembly of TCP on the surface of *V. cholerae*.

In classical strains of *V. cholerae*, expression of cholera toxin and TCP is strongly regulated by environmental signals, such as pH, temperature, amino acid concentration, and osmolarity (19, 20). Coordinate regulation of expression of these virulence factors depends on a transmembrane DNA-binding protein, ToxR, which is encoded in an operon with a second regulatory protein, ToxS (6, 17, 18, 20). ToxR and ToxS are necessary for transcription of a gene encoding an important outer membrane protein of *V. cholerae*, *ompU* (5), as well as a gene encoding a second regulatory protein, *toxT* (7, 10, 22). ToxT belongs to the AraC class of transcriptional regulatory proteins

and activates transcription of the *ctxAB* and *tcpA* operons. The *toxT* gene is contained within the *tcpA* gene cluster itself, and transcription of *toxT* occurs both from a promoter immediately upstream of *toxT* and also as part of the *tcpA* operon, from a longer transcript which originates at the *tcpA* promoter (1, 9).

Several groups have recently shown that two additional regulatory proteins, TcpP and TcpH, are necessary for transcription from the *toxT* promoter and have presented a model in which ToxRS and TcpPH interact to activate *toxT* transcription (4, 8). The genes for *tcpP* and *tcpH* are located immediately upstream of *tcpA* and are transcribed together as an operon (4, 23, 27). The *tcpI* gene is transcribed divergently from *tcpPH*, and TcpI has previously been suggested to be a negative regulator of cholera virulence gene expression (27), although the mechanism of this negative regulation has not yet been elucidated. Expression of the *tcpPH* operon in classical strains of *V. cholerae* is regulated by the same environmental conditions that regulate expression of cholera virulence factors, suggesting that the expression of *tcpPH* couples these environmental signals to transcription of *toxT* and expression of the virulence genes (4). The environmental conditions that induce expression of cholera virulence genes in classical strains of *V. cholerae* (30°C and pH 6.5) are termed ToxR-inducing conditions, whereas the environmental conditions of 37°C and pH 8.5 repress expression of the ToxR regulon (19).

In El Tor strains of *V. cholerae*, expression of cholera virulence factors does not occur in vitro in ToxR-inducing conditions but rather requires special growth conditions termed AKI conditions (11). In AKI conditions, *V. cholerae* are grown statically at 37°C for 4 h and then shifted to overnight shaking at 37°C; these conditions lead to expression of cholera virulence factors in both classical and El Tor strains. Both *toxT* and *tcpPH* are necessary for expression of cholera virulence factors in El Tor strains of *V. cholerae*, as for classical strains.

We have recently shown that transcription of *tcpPH* in El Tor strains of *V. cholerae* cannot be detected by Northern blotting at mid-log-phase growth in ToxR-inducing conditions

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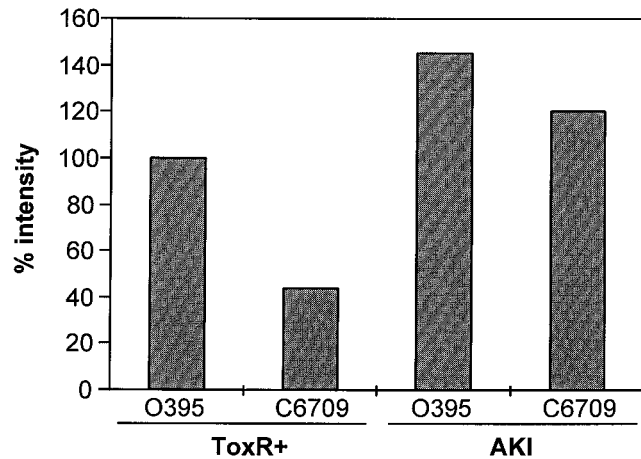


FIG. 2. Quantitation by densitometry of primer extension products of classical (O395) and El Tor (C6709) *tcpPH* transcripts at mid-log-phase growth in ToxR-inducing conditions (ToxR+) and at the end of the static phase in AKI conditions.

(data not shown). Of note, the  $-10$  boxes of the *tcpPH* promoters differed by 1 bp between the classical and El Tor strains, and the  $-35$  box in the El Tor strain was spaced 1 bp further away from the  $-10$  box than in the classical strain (Fig. 1A).

The *tcpI* transcriptional start site was similarly mapped. A 90-bp primer extension product was obtained from O395 RNA purified from cells grown to mid-log phase in ToxR-inducing conditions with oligonucleotide YM8 (data not shown). This corresponds to a transcriptional start site at a cytosine residue 241 bp upstream of the *TcpI* translational start codon (denoted by an asterisk in Fig. 1A). This start site was confirmed by using oligonucleotides YM13 and YM14, residing 50 nucleotides on either side of YM8. With YM8, identical products were obtained from O395 RNA purified from cells grown in AKI conditions as well as from C6709 RNA purified from cells grown in ToxR-inducing and AKI conditions (data not shown); no primer extension products were found in either biotype when grown in ToxR-repressing conditions. The putative  $-10$  boxes of the *tcpI* promoters differed by 2 bp between the two biotypes, and we did not find an obvious  $-35$  box in either biotype (Fig. 1A). Note that the transcription start sites and putative promoter regions for both *tcpI* and *tcpPH* differed from those reported previously (23).

**Quantitating transcription of *tcpPH* in classical and El Tor strains by primer extension.** We used primer extension to quantitate transcription of *tcpPH* in the two biotypes in ToxR-inducing and AKI conditions (Fig. 2). Primer extension was carried out as described above, and the products were visualized by autoradiography. Densitometry analysis was performed by scanning the radiographs and performing analysis with a Power Macintosh G3 computer using the public-domain NIH Image Program (version 1.61) (developed at the National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). At the mid-log phase of growth, the classical *V. cholerae* strain O395 had approximately twice as much *tcpPH* transcript as did the El Tor biotype, while both strains had equivalently high levels of transcription of *tcpPH* during growth in AKI conditions (Fig. 2).

**Construction of *tcpPH::gusA* transcriptional fusions and quantitation of expression of *tcpPH* in each biotype during growth in ToxR-inducing conditions.** To better quantitate ex-

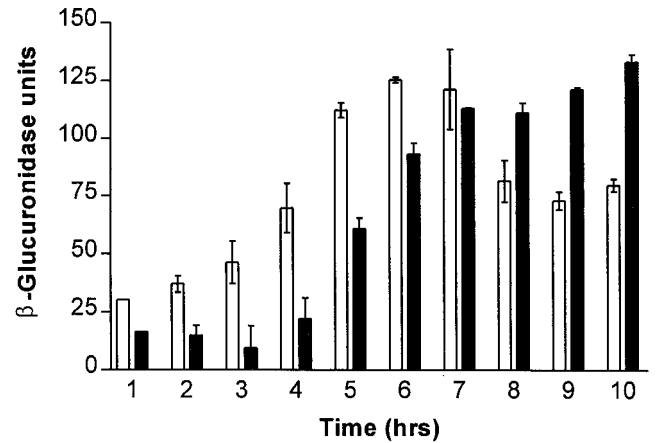


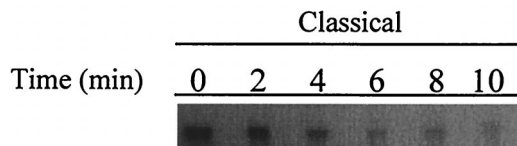
FIG. 3. Assays of *tcpPH::uidA* promoter fusion activities during growth in ToxR-inducing conditions. Error bars represent standard errors of the mean for quadruplicate measurements. Results for the classical promoter fusion strain YM2-34 are shown as open bars, and those for the El Tor promoter fusion strain YM2-35 are shown as solid bars.

pression of the *tcpPH* promoter in each biotype over the course of growth, we constructed transcriptional fusions between the *tcpPH* promoters and the *gusA* gene, encoding  $\beta$ -glucuronidase. We recovered the *tcpI-tcpP* intergenic region, containing the putative *tcpPH* promoters, from both biotypes by PCR (Fig. 1A) and cloned these fragments into plasmid pUJ10, then replaced the *phoA* gene in each with the *uidA* gene, encoding  $\beta$ -glucuronidase. This resulted in plasmids pYM2-25 (classical *tcpPH* promoter) and pYM2-24 (El Tor *tcpPH* promoter). The *tcpPH* promoter-*uidA* fragments were then recovered from these plasmids by digestion with *XbaI* and *NotI* and cloned into a variant of plasmid p6891MCS, which contains a multiple cloning site within a fragment of the *V. cholerae lacZ* gene (2) and the *rmbB* transcriptional terminator from plasmid pKK223-3 (positioned to be 5' of the *tcpPH* promoter in each plasmid). This yielded plasmids pYM2-27 (classical *tcpPH::uidA* fusion) and pYM2-26 (El Tor *tcpPH::uidA* fusion). Each *tcpPH::uidA* fusion was then exchanged by double homologous recombination into the *lacZ* gene of the respective *V. cholerae* host, as previously described (3), resulting in strains YM2-34 (classical) and YM2-35 (El Tor).

$\beta$ -Glucuronidase assays were performed as described previously with some modifications (12). Briefly, strains YM2-34 and YM2-35 were grown overnight and then back-diluted into 5 ml of LB medium and grown in ToxR-inducing conditions. At various time points, 500  $\mu$ l of bacterial culture was centrifuged and washed once with 50 mM phosphate buffer (pH 7.0). Washed cells (50  $\mu$ l) were lysed by adding 10  $\mu$ l of toluene and vortexing for 20 s. Eight hundred fifty microliters of 50 mM phosphate buffer was added to the lysed cells, and samples were incubated for 10 min at 37°C. *p*-Nitrophenyl- $\beta$ -D-glucuronide (100  $\mu$ l; Sigma, St. Louis, Mo.) was added, and the samples were incubated at 37°C until they developed a yellow color, at which point reactions were stopped by the addition of 400  $\mu$ l of 3 M 2-amino-2-methylpropanediol (Sigma). Samples were vortexed for 20 s and centrifuged for 5 min, and the absorbance of the supernatants at 420 nm was measured.

As shown in Fig. 3, the *tcpPH::uidA* fusion in the classical background demonstrated increased transcription from the *tcpPH* promoter by 4 h in ToxR-inducing conditions, peaking at 6 h and decreasing thereafter. In contrast, transcription from the *tcpPH* promoter in the El Tor background began later,

A.



B.

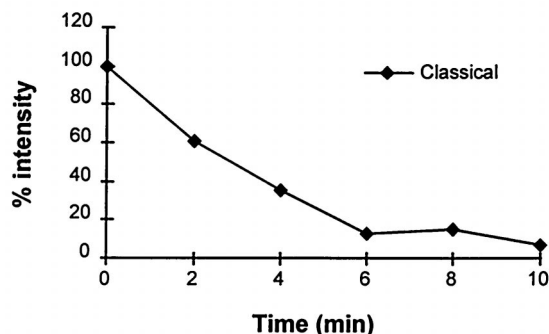


FIG. 4. Estimation of *tcpPH* RNA stability after addition of rifampin, as measured by primer extension analysis and densitometry analysis. (A) Primer extension results after addition of rifampin at time zero for the classical strain. (B) Plot of densities of primer extensions results from panel A.

increasing after 5 h and not peaking until 10 h. As seen previously (Fig. 2), transcription of *tcpPH* in the classical biotype was substantially higher than in the El Tor biotype at mid-log-phase growth in ToxR-inducing conditions. However, transcription of *tcpPH* in the El Tor biotype was greater than in the classical biotype during late-log-phase and stationary-phase growth, even though previous results suggest that little or no cholera toxin or TCP is expressed after overnight growth in ToxR-inducing conditions in the El Tor biotype (21).

**Assessment of *tcpPH* RNA stability in each biotype.** One possibility is that the *tcpPH* transcript is less stable in the El Tor strain, even though initiation of transcription is seen in late-log-phase or stationary-phase growth. The stability of the *tcpPH* message was compared between the classical and El Tor biotypes using rifampin as previously described (28). In brief, cultures were grown to mid-log phase in ToxR-inducing conditions, rifampin was added (200  $\mu$ g/ml, final concentration), and RNA was harvested at various times following the addition of rifampin. RNA was quantitated spectrophotometrically and visualized by using ethidium bromide and agarose gel electrophoresis. Twenty micrograms of RNA was used for primer extension analysis as described above. One half of the total primer extension product was loaded onto a 6% denaturing polyacrylamide gel and separated by electrophoresis. Products were visualized by autoradiography, and band intensities were determined as above and compared with the zero timepoint.

The intensity of the *tcpPH* transcripts at mid-log-phase growth in ToxR-inducing conditions was greater for the classical than the El Tor biotype strain prior to the addition of rifampin, as seen above (data not shown). The half-life of the *tcpPH* message in the classical biotype was approximately 2 min (Fig. 4). This short half-life is similar to that reported previously for the *toxT* transcript (30). Although the faint primer extension signal seen with the El Tor biotype prevented a comparably accurate measurement, the half-life of the *tcpPH*

transcript in this biotype also approximated 2 min, no different from the classical strain (data not shown).

Yu et al. have previously shown in a classical strain of *V. cholerae* that TcpPH and ToxR are required for transcription from the promoter immediately upstream of *toxT* in early- to mid-log-phase growth in ToxR-inducing conditions but that transcription from this promoter begins to diminish 5 to 9 h after back-dilution of a culture and is undetectable after 9 h (30). Later in growth, they showed that transcription of *toxT* occurs from the upstream *tcpA* promoter as part of an autoregulatory loop primed by the initial transcription from the *toxT* promoter. If no *toxT* transcription occurred early in growth, then no cholera toxin was produced.

Medrano et al. examined transcription of *toxT* in an El Tor strain of *V. cholerae* in AKI conditions (15). They demonstrated that ToxR-dependent transcription from the *toxT* promoter was seen at the end of 4 h of static growth but was not seen after a shift to shaking growth conditions. During shaking growth, only the longer *toxT* transcript from the *tcpA* promoter was seen, as part of an autoregulatory loop. If the cultures were kept in static growth, the initial transcription from the *toxT* promoter disappeared, and no cholera toxin (and presumably no transcription from the *tcpA* promoter) was seen later in growth. This suggests that the temporal sequence of transcription from the *toxT* and *tcpA* promoters is important to the expression of cholera virulence genes.

Kovacikova et al. used a *tcpP::lacZ* fusion in an El Tor strain of *V. cholerae* to show that transcription of *tcpPH* was minimal at mid-log-phase growth in ToxR-inducing conditions but was much more abundant in AKI conditions (14). We have previously shown an absence of *tcpPH* mRNA by Northern blot in an El Tor strain of *V. cholerae* at mid-log-phase growth in ToxR-inducing conditions (21).

Utilizing the more sensitive assays here, we now show that the *tcpPH* message is markedly reduced but not absent at mid-log-phase growth in an El Tor strain of *V. cholerae* in ToxR-inducing conditions but that transcription is turned on later in growth. This suggests the possibility that failure of *tcpPH* transcription early in growth in ToxR-inducing conditions in the El Tor biotype of *V. cholerae* (compared to the classical biotype) may fail to activate the autoregulatory loop from the *tcpA* promoter that is necessary for continued expression of *toxT* and that this may abrogate expression of cholera toxin and TCP. An early peak of *tcpPH* (and *toxT*) transcription may be necessary for the later expression of cholera toxin and TCP, and the transcription of *tcpPH* seen later in the El Tor biotype, after cells have entered the late log phase of growth, may not be sufficient to activate expression of cholera virulence factors. This model suggests that the difference in expression of cholera virulence factors between the El Tor and classical biotypes of *V. cholerae* may be related to the timing of transcription of *tcpPH* during ToxR-inducing conditions rather than the absolute levels of transcription.

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