RNA thermometer controls temperature-dependent virulence factor expression in *Vibrio cholerae*

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Vibrio cholerae is the bacterium that causes the diarrheal disease cholera. The bacteria experience a temperature shift as *V. cholerae* transition from contaminated water at lower temperatures into the 37 °C human intestine. Within the intestine, *V. cholerae* express cholera toxin (CT) and toxin-coregulated pilus (TCP), two main virulence factors required for disease. CT and TCP expression is controlled by the transcriptional activator protein ToxT. We identified an RNA thermometer motif in the 5′ UTR of *toxT*, with a fourU anti-Shine-Dalgarno (SD) element that base pairs with the SD sequence to regulate ribosome access to the mRNA. RNA probing experiments demonstrated that the fourU allowed access to the SD sequence at 37 °C but not at 20 °C. Moreover, mutations within the fourU element (U5C, U7C) that strengthened base-pairing between the anti-SD and SD sequences prevented access to the SD sequence even at 37 °C. Translation of ToxT-FLAG from the native *toxT* UTR was enhanced at 37 °C, compared with 25 °C in both *Escherichia coli* and *V. cholerae*. In contrast, the U5C, U7C UTR prevented translation of ToxT-FLAG even at 37 °C. *V. cholerae* mutants containing the U5C, U7C UTR variant were unable to colonize the infant mouse small intestine. Our results reveal a previously unknown regulatory mechanism consisting of an RNA thermometer that controls temperature-dependent translation of *toxT*, facilitating *V. cholerae* virulence at a relevant environmental condition found in the human intestine.

T he aquatic, Gram-negative bacterium *Vibrio cholerae* is responsible for the diarrheal disease cholera. Cholera has swept through global human populations in large pandemics, with the first six pandemics being caused by the O1 classical biotype, and the seventh current pandemic caused by the O1 El Tor biotype. The organisms infect humans through the consumption of contaminated water sources. Once *V. cholerae* is inside the human small intestine, the transcription factor protein ToxT directly activates the *ctx* and *tcp* genes, which encode the essential virulence factors cholera toxin (CT) and toxin-coregulated pilus (TCP), respectively (1). TCP is required for intestinal colonization, and CT induces the profuse watery diarrhea that is the hallmark of this disease (2, 3). *V. cholerae* strains that lack *toxT* are unable to express CT or TCP and are unable to cause disease (4). A regulatory cascade that incorporates input from various environmental signals, the ToxR regulon, controls the transcription of *toxT* to ensure maximal expression within the intestine (1, 5). Additionally, ToxT-dependent transcriptional activity is controlled by environmental signals found within the intestine (6, 7). We report here a third mechanism that controls virulence factor expression in *V. cholerae* via temperature-dependent modulation of the translation of ToxT.

RNA thermometers are temperature-sensing RNA sequences found in the 5′ UTR of mRNA (8). The RNA thermometer folds into a structure to prevent access of the ribosome to the Shine-Dalgarno (SD) sequence within the mRNA at lower temperatures. As temperature increases the structure unfolds and facilitates mRNA translation at elevated temperatures. One type of RNA thermometer is the fourU element, which is characterized by a UUUU motif that base pairs with the SD sequence to form a stem-loop structure that unfolds by a zipper mechanism as temperature increases (9). The stem-loop can be stabilized by

strengthening the base pairing between the fourU element and SD sequence through the substitution of G-C base pairs, and this results in an elevated melting temperature (10). The best-characterized fourU thermometer controls temperature-dependent translation of a small heat shock protein, AgsA, in *Salmonella enterica* (11). FourU thermometers also control temperature-dependent translation of the virulence factor LcrF in *Yersinia* spp. (12) and iron-acquisition genes in *Shigella dysenteriae* and *Escherichia coli* (13), ensuring appropriate expression of factors important for virulence within host tissues. Other types of RNA thermometers control virulence gene expression in *Listeria monocytogenes* (14) and *Neisseria meningitidis* (15).

Here we show that *toxT* translation in *V. cholerae* is controlled in a temperature-dependent manner by a fourU element within the 5′ UTR. At human body temperature, the thermometer structure within the mRNA opens to allow ToxT translation and subsequent virulence factor expression. Mutations within the fourU element that strengthen the secondary structure and prevent melting at 37 °C also prevent virulence of *V. cholerae* within an animal model for cholera, indicating that the RNA thermometer is an important modulator of *V. cholerae* virulence.

Results

The *toxT* 5′ UTR Contains a FourU RNA Thermometer Element. An increase of temperature to 37 °C signifies entry into a mammalian host to a pathogenic bacterium, and temperature-dependent virulence factor expression has been shown to be regulated by RNA thermometers in *Yersinia* spp. (12) and *Listeria monocytogenes* (14). We identified a potential RNA thermometer in the 5′ UTR of *V. cholerae toxT* that has characteristics of a fourU element (Fig. 1*A*). The Mfold RNA structure prediction program (16) predicted that the four uridine residues in the mRNA at positions +5 to +8

Significance

Vibrio cholerae causes the life-threatening diarrheal disease cholera. These bacteria live in aquatic environments and are introduced into humans through contaminated water sources. The bacteria express virulence factors upon entry into the human gastrointestinal tract. The present study shows that virulence factor expression is controlled by the shift in temperature to 37 °C, through an RNA thermometer that controls translation of the major virulence regulator protein ToxT. This simple mechanism allows maximal virulence factor expression within the mammalian host and may be more widespread among human pathogens than currently known.

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with respect to the transcription start site base pair with the SD ribosome binding site at residues +56 to +59. This forms a stem-loop structure containing canonical and noncanonical base-pairing between the fourU element and the SD sequence that is characteristic of an RNA thermometer (11).

To determine whether this UTR could impart temperature-dependent control over translation, we created a translational fusion of the *toxT* 5′ UTR to *lacZ* in pBAD18-*lacZ*481 (12). High-level transcription was induced by the addition of arabinose, and we measured translation at 25 °C and 37 °C by determining β-galactosidase levels in two different *V. cholerae* O1 biovars, classical (strain O395) and El Tor (strain C6706), as well as in *E. coli* (strain DH5α) (Fig. 1*B*). In both *V. cholerae* strains

as well as in *E. coli*, translation from the native *toxT* UTR (WT) was ~150% greater at 37 °C compared with 25 °C, indicating the presence of an RNA thermometer in the UTR.

The noncanonical U-G base pairs in the fourU RNA thermometer can be strengthened by substituting a C residue in place of the U residue(s), which raises the melting temperature of the fourU-SD sequence double-stranded stem (10). Using site-directed mutagenesis we created *toxT* 5′ UTRs with U5C, U7C, and U5C U7C (U5, 7C). These mutant UTRs were fused to *lacZ* in pBAD18 as above and measured for β-galactosidase activity at 25 °C and 37 °C in *V. cholerae* and *E. coli* in the presence of arabinose (Fig. 1*B*). The U5C substitution caused a dramatic reduction in LacZ translation at 37 °C in both *V. cholerae* strains and *E. coli* (95–98%) compared with translation from the native UTR. In contrast, the U7C substitution led to only modest reductions (25–60%) in LacZ translation at 37 °C compared with the native UTR. These results are consistent with the zipper-type unfolding model of fourU thermometer melting (9), because the U5C mutation would be predicted to stabilize the entire stem-loop structure, whereas the U7C mutation would not. The U5, 7C double mutation led to a near absence (96–98% reduction) of detectable LacZ translation at 37 °C in all three bacterial strains.
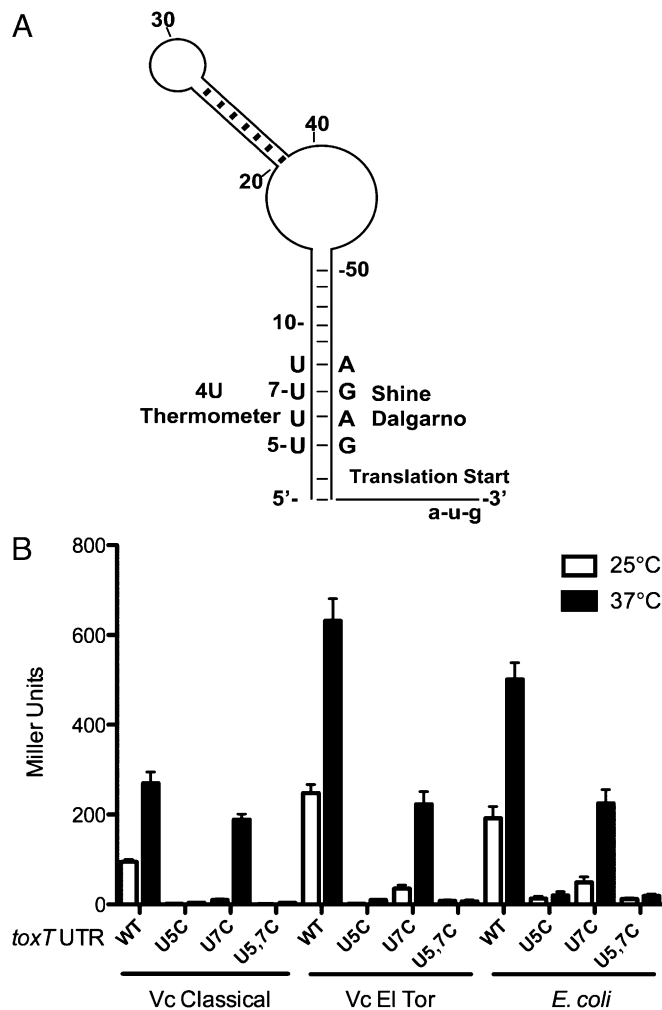
RNA Structure Probing Reveals Melting of *toxT* UTR Thermometer at Elevated Temperature. The secondary structures of 5′-labeled *toxT* UTRs, both the native UTR and the U5, 7C mutant UTR, were probed at increasing temperatures using RNase T1 (cuts 3′ of single-stranded guanines) and RNase V1 (specific for double-stranded regions). The cleavage products were separated on a denaturing 15% polyacrylamide gel. Focusing on the SD region of the native *toxT* UTR, T1 cleavage revealed that the SD region (G57, G59) becomes single-stranded as the temperature increases from 20 °C to 37 °C (Fig. 2*A*; the entire UTR structure can be found in Fig. S2). In contrast, no melting of the SD region of the U5, 7C *toxT* UTR occurs even at the elevated temperature of 37 °C, as evidenced by lack of cleavage of G57 and G59 by RNase T1. These results are consistent with the lack of translation from the U5, 7C UTR at 37 °C as shown above (Fig. 1*B*), being due to lack of melting of the RNA thermometer.

The *toxT* native and U5, 7C UTRs were also probed with RNase V1 to detect double-stranded regions (Fig. 2*B*). Conditions were chosen that highlighted differences between these RNA structures. RNase V1 probing revealed that the region between G59 and G62, which includes the lower part of the SD sequence and stem-loop structure, became more obviously double-stranded in the U5, 7C mutant UTR at increasing temperature up to 37 °C. In contrast, under identical conditions, no enhanced double-stranded base pairs could be detected in this region with increasing temperature in the native *toxT* UTR. These results complement the RNase T1 cleavage patterns and indicate that the native UTR thermometer melts at 37 °C, whereas the U5, 7C thermometer remains clamped in a double-structure at higher temperatures.

The FourU Thermometer Mediates Temperature-Dependent Translation of ToxT. Transcription of *toxT* within *V. cholerae* is complex and is subject not only to many environmental cues feeding into the ToxR- and TcpP-dependent transcription of *toxT* (5) but also to an autoregulatory loop whereby ToxT activates its own transcription from the *tcpA* promoter (17). To avoid these overlapping regulatory mechanisms that converge on *toxT* transcription and focus instead on temperature-dependent translation of ToxT, we expressed *toxT* with its native 5′ UTR from the arabinose-inducible pBAD promoter within plasmid pBAD18 (18). A FLAG tag was added to ToxT to facilitate detection by Western immunoblot with anti-FLAG antibody. As a control for temperature-independent translation, we also created a plasmid that expresses *toxT*-FLAG from the arabinose-inducible pBAD promoter but with the 5′ UTR from *araBAD* [present in plasmid pBAD24 (18)] replacing the native *toxT* 5′ UTR.

When *toxT* is transcribed at high levels in *E. coli* from the pBAD promoter in the presence of arabinose, translation of ToxT-FLAG

Fig. 1. The *toxT* 5′ UTR modulates temperature-dependent translation of LacZ. (*A*) Schematic of the predicted structure of the *toxT* 5′ UTR. The secondary structure was derived from Mfold (16). Shown are the relevant nucleotides within the putative fourU thermometer and the SD sequence; the complete nucleotide sequence and structure is shown in Fig. S1. The start codon and the U5 and U7 residues are indicated. (*B*) Effect of temperature on LacZ translation from *toxT* 5′ UTR. The *toxT* 5′UTR-*lacZ* (WT) construct, and the U5C, U7C, and U5C U7C (U5, 7C) mutant *toxT* 5′ UTR-*lacZ* constructs were expressed from the pBAD promoter in plasmids pBO1296, pBO1826, pBO1829, and pKEK1807, respectively. Plasmids were transformed into *V. cholerae* O1 strains O395 (Classical), C6706 (El Tor), and *E. coli* strain DH5α, and strains assayed for β-galactosidase activity during midlog growth (OD$_{600}$ of 0.4–0.5) at the temperatures indicated in the presence of 0.1% arabinose. Translation from the WT UTR was significantly greater at 37 °C than at 25 °C (*P* < 0.01), and translation from WT UTR was significantly greater than from U5C and U5,7C UTRs at 37 °C (*P* < 0.001); Student's two-tailed *t* test.

from the native *toxT* UTR is temperature-dependent (Fig. 3*A*). Low levels of ToxT-FLAG could be detected at 25 °C, with more ToxT-FLAG detectable at 30 °C and maximum amounts of ToxT-FLAG detectable at 37 °C and 42 °C. When *toxT-FLAG* is transcribed at high levels in *V. cholerae* from the pBAD promoter in the presence of arabinose, translation of ToxT-FLAG from the native *toxT* UTR was even more strongly temperature-dependent (Fig. 3*A*). The *toxT-FLAG* transcript with the native *toxT* UTR was expressed in both *V. cholerae* biotypes, classical and El Tor, and these strains additionally carried chromosomal deletions in *toxT*, to avoid any potential complications due to the presence of the chromosomally encoded ToxT. In both classical and El Tor biotypes, no ToxT-FLAG was detectable at 25 °C, modest amounts of ToxT-FLAG could be detected at 30 °C, and maximum amounts of ToxT-FLAG were detectable at 37 °C and 42 °C. [ToxT is known to undergo proteolytic degradation, and a smaller ToxT degradation product could also be detected in these assays (19).]

Temperature-dependent translation is specifically due to the presence of the *toxT* 5′ UTR, because when *toxT-FLAG* was instead transcribed from the same promoter with the *araBAD* 5′ UTR replacing the native *toxT* UTR, similar levels of ToxT-FLAG were translated at all temperatures in *E. coli* and both *V. cholerae* biotypes (Fig. 3*B*). These results demonstrate that the native *toxT* 5′ UTR acts as an RNA thermometer to control temperature-dependent translation of ToxT.

Strengthening Base-Pairing Within the FourU Thermometer Inhibits ToxT Translation at Elevated Temperatures. The mutant *toxT* UTRs with strengthened stem-loop binding (U5C, U7C, and U5, 7C) were also fused to *toxT-FLAG*, and these transcripts were expressed from the pBAD promoter in *E. coli* at 37 °C (Fig. 3*C*). As was seen above, the native *toxT* UTR allows translation of ToxT-FLAG at 37 °C. The introduction of the U5C or the U7C mutation into the *toxT* UTR results in less ToxT-FLAG translation, whereas the introduction of both U5C and U7C mutations (U5, 7C) results in no detectable ToxT-FLAG translation. These results are consistent with the RNA probing experiments above, in which the U5, 7C
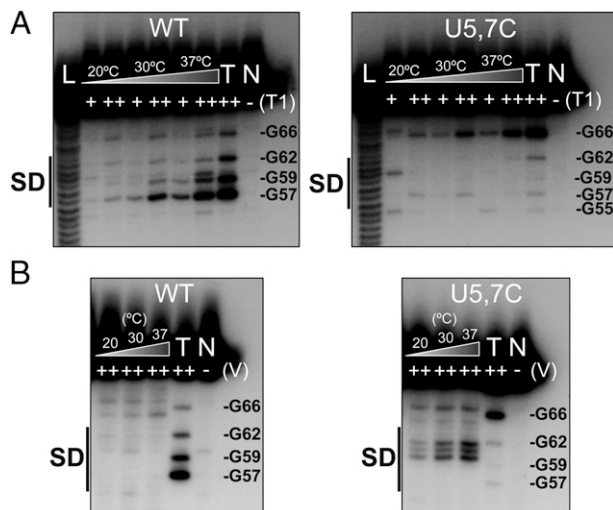

Fig. 3. The *toxT* 5′ UTR fourU RNA thermometer facilitates temperature-dependent translation of ToxT. (*A*) Schematic representation of the 5′-*toxT* UTR-*toxT*-FLAG transcript of pKEK1733. *E. coli* DH5α (*E. coli*), *V. cholerae* O1 Δ*toxT* strains KKV2426 (*Vc* El Tor) and KKV2288 (*Vc* Classical) carrying plasmid pKEK1733 were grown at the indicated temperatures in the presence of 0.1% arabinose. (*B*) Schematic representation of the 5′-*araBAD*-UTR-*toxT*-FLAG transcript of pKEK1789. *E. coli* DH5α (*E. coli*), *V. cholerae* O1 strain C6706 (Vc El Tor), and *V. cholerae* O1 strain O395 (Vc Classical) carrying plasmid pKEK1789 (5′-*araBAD*-UTR) were grown at the indicated temperatures in the presence of 0.1% arabinose. Western immunoblot using anti-FLAG monoclonal antibody was performed in *A* and *B* with whole-cell lysates (OD$_{600}$ = 0.6). (*C*) Mutations in the fourU thermometer disrupt temperature-dependent translation of ToxT. *E. coli* DH5α carrying plasmids pKEK1733 (5′-*toxT* UTR), pKEK1734 (5′-U5C *toxT* UTR), pKEK1735 (5′-U7C *toxT* UTR), or pKEK1736 (5′-U5C U7C *toxT* UTR) were grown at 37 °C in the presence of 0.1% arabinose. Western immunoblot using anti-FLAG monoclonal antibody was performed with whole-cell lysates (OD$_{600}$ = 0.6).

UTR remained double-stranded at 37 °C, which would sequester the SD sequence and prevent ToxT translation.

The FourU Thermometer Controls Virulence Factor Expression in *V. cholerae* in Vitro. As mentioned above, a complicated regulatory cascade controls transcription of *toxT* that could mask the effects of temperature-dependent translation of ToxT. To determine whether the fourU thermometer controls temperature-dependent virulence factor expression, we transcribed *toxT* with either native or mutant 5′ UTR from the pBAD promoter in *V. cholerae* O1 classical and El Tor strains that contained deletions of the chromosomal *toxT* gene, during growth at 25 °C and 37 °C. All cultures were assayed at the same stage of midlog growth (OD$_{600}$ of 0.6). Transcript levels of *toxT* were measured by quantitative RT-PCR, which demonstrated that the amount of *toxT* transcripts was similar at both temperatures for the wild-type and mutant UTR-*toxT* transcripts in both classical and El Tor *V. cholerae*

Fig. 2. The *toxT* 5′ UTR fourU RNA thermometer facilitates temperature-dependent access to the SD sequence. Enzymatic cleavage of 5′ ³²P-end-labeled *toxT* RNA was carried out at 20 °C, 30 °C, and 37 °C. RNA fragments were separated on 15% polyacrylamide. (*A*) RNase T1 (cuts 3′ of single-stranded guanines) was used at the indicated concentrations [0.01 U (+) and 0.1 U (++)]. (*B*) RNase V1 (cuts double-stranded, stacked regions) was used at 0.1 U. Lane L is the alkaline ladder, lane N contains no RNase, and RNA was cleaved with 0.1 U T1 at 42 °C in lane T. WT indicates the native *toxT* 5′ UTR, and U5, 7C indicates the U5C U7C *toxT* 5′ UTR. The SD sequence is labeled, and select nucleotides are noted.
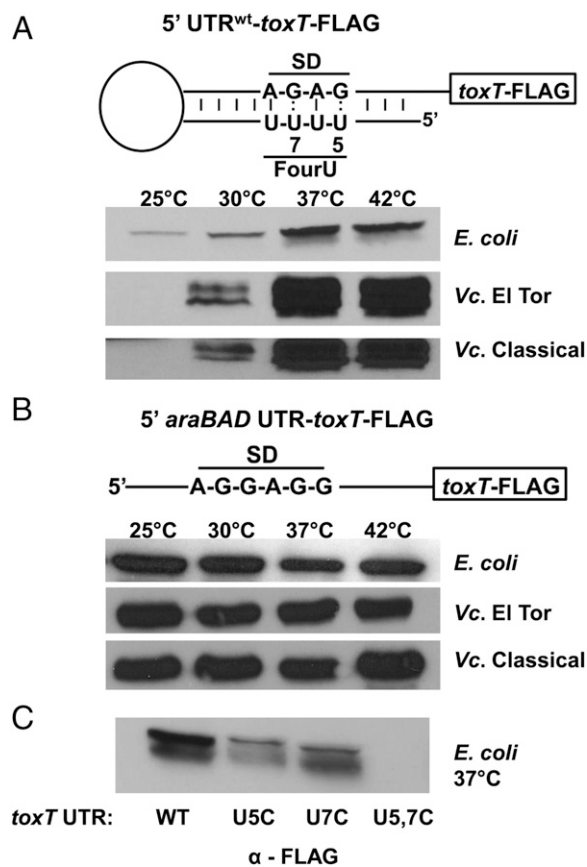
(Fig. 4A; no significant difference in expression in any sample; Student *t* test).

We also measured transcript levels of the ToxT-dependent virulence gene *ctxB* in the same strains grown at 25 °C and 37 °C (Fig. 4B). When normalized to transcript levels of these genes at 25 °C in the same strain expressing the native UTR-*toxT* gene, it could be

Fig. 4. The *toxT* 5′ UTR fourU RNA thermometer facilitates temperature-dependent expression of CT. *V. cholerae* O1 Δ*toxT* strains KKV2288 (Classical) and KKV2426 (El Tor) carrying plasmids pKEK1682 (5′-*toxT* UTR-*toxT*), pKEK1683 (5′-U5C *toxT* UTR-*toxT*), pKEK1685 (5′-U7C *toxT* UTR- *toxT*), or pKEK1686 (5′-U5C U7C *toxT* UTR- *toxT*) were grown at 25 °C and 37 °C in the presence of 0.1% arabinose and harvested at identical cell densities (OD$_{600}$ = 0.6). mRNA abundance for (A) *toxT* and (B) *ctxB* were determined by quantitative RT-PCR (*Materials and Methods*). (C) Supernatants were assayed for CT.

confirmed that (*i*) *ctx* transcription was higher at 37 °C than at 25 °C in strains expressing the native UTR-*toxT*, ($P < 0.001$ classical and $P < 0.01$ El Tor), (*ii*) the U5C and U5C U7C UTRs caused significant decreases in *ctx* transcription ($P < 0.001$ at 37 °C for both classical and El Tor), and (*iii*) the U7C UTR had little effect on *ctx* transcription ($P < 0.05$ classical 37 °C, no significant difference El Tor). Similar results were seen when transcript levels of the ToxT-dependent virulence gene *tcpB* were measured in the same strains (Fig. S3). These results are consistent with the function of the RNA thermometer in the *toxT* UTR controlling virulence gene transcription by modulating ToxT translation.

Finally, we measured CT expression in these same strains during growth at 25 °C and 37 °C (Fig. 4C). As anticipated, CT expression mirrored *ctxB* transcription in these strains. Under these conditions, CT expression was strongly temperature-dependent in the O1 classical *V. cholerae* strain translating ToxT from the native UTR, with little CT expression at 25 °C, and 30-fold higher CT expressed at 37 °C. CT expression was also temperature-dependent in the O1 El Tor *V. cholerae* strain translating ToxT from the native UTR, with twofold higher CT expressed at 37 °C than at 25 °C. The introduction of the U7C mutation into the UTR caused a modest reduction in CT expression at 37 °C in the classical strain and had no effect on CT expression in the El Tor strain. In contrast, introduction of the U5C mutation into the UTR resulted in no detectable CT expression at either temperature in both the classical and El Tor strains. Likewise, introduction of both U5C and U7C mutations (U5, 7C) into the UTR resulted in no detectable CT expression at either temperature in both classical and El Tor strains. These results confirm that the *toxT* UTR controls temperature-dependent expression of CT. Moreover, the U5C UTR mutation has a stronger negative effect on virulence factor expression than the U7C mutation, consistent with the results seen with LacZ translation from the various *toxT* UTRs (Fig. 1). These results also suggest there may be additional mechanism(s) controlling ToxT translation that vary between *V. cholerae* biotypes.

The FourU Thermometer Controls Virulence of *V. cholerae* in Vivo. The ability of *V. cholerae* to colonize the infant mouse small intestine is correlated with its ability to cause disease in humans (20). We reasoned that strengthening the fourU thermometer by the introduction of the U5C and/or U7C mutations into the genome of *V. cholerae* would reveal whether the fourU thermometer controlled temperature-dependent virulence in vivo. *V. cholerae* classical O1 strains were constructed with U5C, U7C, and U5C U7C *toxT* UTR mutations, and a *V. cholerae* El Tor O1 strain was constructed with the U5C U7C mutations. We used a competition assay in the infant mouse to determine the relative ability of these *V. cholerae* strains to colonize the infant mouse small intestine.

The U5C *toxT* UTR mutant showed an approximate 10-fold defect in its ability to colonize the infant mouse small intestine [competitive index (C.I.) = 0.13, $P < 0.01$] (Fig. 5A). In contrast, the U7C *toxT* UTR mutant colonized the infant mouse intestine similar to the wild-type strain. These results are consistent with the relative effect these single UTR mutations had upon LacZ/ToxT translation and CT expression at 37 °C, with the U5C mutation having a stronger negative effect than the U7C mutation.

The U5C U7C *toxT* UTR mutations in both classical and El Tor O1 strains rendered the bacteria completely incapable of colonizing the infant mouse intestine (Fig. 5B); no mutant *V. cholerae* strains were recovered from any mice (C.I. $< 5 \times 10^{-5}$; $P < 0.001$). These results are consistent with the inhibitory effect of the U5C U7C mutations on ToxT translation at 37 °C (Fig. 3C), which is due to the lack of melting of the RNA thermometer (Fig. 2) and sequestration of the SD sequence. Our results demonstrate the importance of the RNA thermometer in regulating the virulence of *V. cholerae*.

Discussion

Controlled access to the ribosome binding site (RBS) within mRNA is a mechanism that modulates the rate of protein synthesis in bacteria. RBS access can be controlled by RNA–RNA
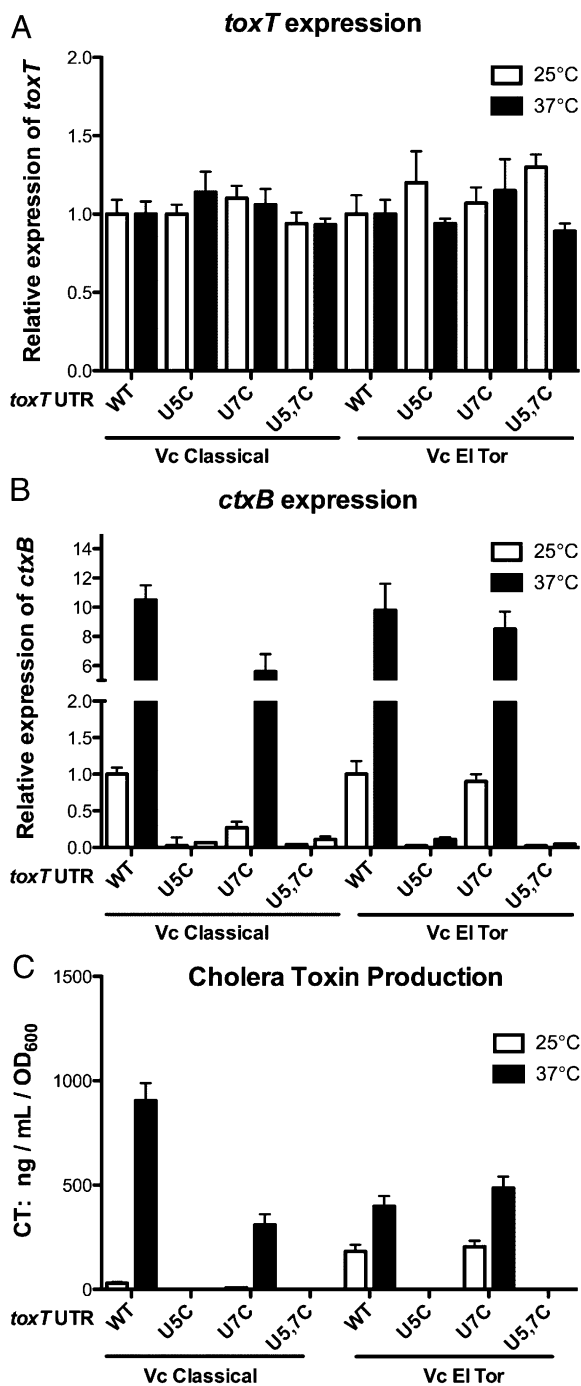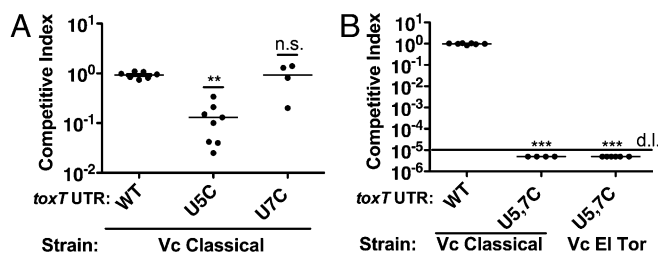
Fig. 5. The *toxT* 5′ UTR fourU RNA thermometer controls virulence of *V. cholerae*. (*A*) *V. cholerae* O1 classical strains KKV598 (WT), KKV2333 (U5C 5′*toxT* UTR), and KKV2368 (U7C 5′*toxT* UTR) were coinoculated with the wild-type strain O395 perorally into infant mice. **P < 0.01, Student *t* test; n.s., not significant. (*B*) *V. cholerae* O1 classical strains KKV598 (WT) and KKV2425 (U5C, U7C 5′*toxT* UTR), and *V. cholerae* O1 El Tor strain KKV2382 (U5C, U7C 5′*toxT* UTR) were coinoculated with the wild-type strains O395 or C6706 perorally into infant mice. No U5C U7C mutant bacteria were recovered from the intestine, detection limit (d.l.) is 5×10^{-5}. ***P < 0.001, Student *t* test. Inocula in *A* and *B* contained a ratio of ~1:1 mutant/wild-type; intestinal homogenates were recovered at 24 h after inoculation and cfu of wild-type and mutant strains determined. The competitive index is given as the output ratio of mutant/wild-type divided by the input ratio of mutant/wild-type; each value shown is from an individual mouse.

interactions, including *trans*-acting small RNAs that bind to the SD sequence and prevent 30S ribosome binding (21). RBS access can also be controlled by *cis*-acting RNA sequences that base pair with the SD sequence and form a secondary structure that prevents ribosome binding. These *cis*-acting sequences include riboswitches that bind diverse ligands and primarily control expression of biosynthetic pathways (22). Another type of *cis*-acting sequence is RNA thermometers, which control temperature-dependent translation of proteins (8). RNA thermometers such as the ROSE element are typically found within the 5′ UTR of heat shock protein genes; ROSE prevents translation until heat shock temperature, by sequestration of the SD sequence through base-pairing (23).

Another type of RNA thermosensor is the fourU element, which has been best characterized in the 5′ UTR of an *S. enterica* heat shock gene, *agsA* (11). The four consecutive uridine residues within this element base pair with the SD sequence and form a stem-loop that prevents ribosome binding at lower temperatures. Critical to the function of this thermometer are two noncanonical base pairs between the uridine residues within the element and G residues in the SD sequence. This thermometer opens upon increased temperature through a zippering mechanism initiated at the base of the stem-loop closest to the translation start (9). Our results have demonstrated that a similar fourU thermometer is present in the 5′ UTR of *toxT* mRNA, and it functions to regulate temperature-dependent ToxT translation in *V. cholerae*. This regulatory mechanism allows for high levels of ToxT at the mammalian host temperature of 37 °C, and low to no ToxT protein at the lower temperatures found in the aquatic environmental reservoir where *V. cholerae* are naturally found. Because ToxT is the direct transcriptional activator of the most important *V. cholerae* virulence factors, CT and TCP, the fourU thermometer controls temperature-dependent induction of virulence as the bacteria move from the aquatic environment into the human host.

Several lines of evidence demonstrated the function of this thermosensor in the 5′ UTR of *toxT*, including direct RNA probing, *lacZ* translational fusions, Western immunoblot for ToxT protein, and measurement of ToxT-dependent virulence factor expression. Moreover, the substitution of C-G base pairs in place of the U-G base pairs (U5C and/or U7C) within the stem-loop structure inhibited or prevented thermal induction of ToxT translation and subsequent virulence, as predicted. On the basis of previous NMR studies (9), the fourU RNA thermometer opens by a zippering mechanism initiating from the base of the stem loop,

and thus the U5C mutation would be expected to act like a clamp and have a stronger negative effect on thermal induction of ToxT translation than the U7C mutation. Indeed, the U7C mutation had only modest effects on ToxT translation and no significant effect on intestinal colonization, whereas the U5C mutation caused significant decreases in ToxT translation and an approximately 10-fold reduction in intestinal colonization. Importantly, the 5′ UTR containing both U5C and U7C mutations prevented ToxT translation and subsequent virulence factor expression at 37 °C, and *V. cholerae* O1 pandemic strains (classical and El Tor) containing these mutations were completely unable to colonize the intestine. RNA probing revealed that the stem-loop structure of the U5C U7C 5′ UTR remained double-stranded at 37 °C, emphasizing the importance of the fourU thermometer in the temperature-dependent virulence of *V. cholerae*.

As *V. cholerae* transition from the aquatic environment into the human intestine, the environment encountered within the small intestine leads to the ToxT-dependent induction of TCP and CT expression and subsequent disease. A number of different environmental signals have been identified that modulate the ToxR- and TcpP-dependent induction of *toxT* transcription, a critical event in this virulence cascade (5), as well as those that modulate the activity of ToxT (6, 7). The increase to 37 °C as the bacteria move into the host seems like an obvious environmental change that signifies optimal disease conditions, and our results demonstrate how the RNA thermometer in the 5′ UTR of *toxT* modulates virulence by enhancing ToxT translation at temperatures found within the intestine. Our studies reveal yet another layer of control over *V. cholerae* pathogenesis that ensures the correct temporal and spatial expression of virulence factors. The RNA thermometer mechanism provides temperature-dependent control over translation from a preexisting mRNA, and thus may allow for quicker or more precise control over virulence gene expression when entering or exiting a host. FourU RNA thermometers have been identified that control virulence by temperature-dependent expression of LcrF in *Yersinia* spp. (12) and iron-acquisition genes in *Shigella dysenteriae* and enteropathogenic *E. coli* (13). RNA thermometers represent a simple yet elegant mechanism for controlling temperature-dependent gene expression and may be more widespread among pathogenic bacteria that interact with mammalian hosts than is currently known.

Materials and Methods

Bacterial Strains and Plasmids. *E. coli* strains DH5α (24) and Top10 (Invitrogen) were used for cloning, and SM10λpir was used for conjugation. *V. cholerae* O1 strains O395 [classical biotype (25)] and C6706 [El Tor biotype (26)] were used. *V. cholerae* strain KKV2426 (C6706 Δ*toxT*::Cm) was created via chitin transformation (27) using genomic DNA from strain SY1002 (28). *V. cholerae* strains KKV2333 (O395 *toxT* 5′ UTR U5C), KKV2368 (O395 *toxT* 5′ UTR U7C), KKV2425 (O395 *toxT* 5′ UTR U5C, U7C), KKV2382 (C6706 *toxT* 5′ UTR U5C, U7C), as well as KKV598 (O395 native *toxT* 5′ UTR) and KKV2372 (C6706 native *toxT* 5′ UTR), were constructed by using plasmids pKEK1445, pKEK1468, pKEK1469, and pKEK1808 to introduce the mutations into *V. cholerae* strains KKV2288 (29) and KKV2426 by allelic exchange. A complete list of primers and plasmids used is provided in Tables S1 and S2.

Strains were grown in Luria broth (LB) at the indicated temperatures. Antibiotics were used at the following concentrations: ampicillin (Ap), 100 μg/mL; chloramphenicol (Cm), 2 μg/mL; streptomycin (Sm), 100 μg/mL and 1 mg/mL. When required, arabinose was added to the media at a concentration of 0.1%. Plasmids used are listed in Table S2, and details on their construction are included in SI Materials and Methods. All constructs were verified by sequencing.

β-Galactosidase Assays and Western Immunoblot. For β-galactosidase assays, overnight cultures were diluted 1:100 in LB plus 100 μg/mL ampicillin and 0.1% arabinose, grown at the indicated temperatures to an optical density at 600 nm of 0.3–0.8, permeabilized with chloroform and SDS, and then assayed for enzymatic activity by the method of Miller (30). All assays were performed independently at least twice, with triplicate samples. For Western immunoblots, strains were grown overnight in LB + ampicillin (Amp) at 37 °C. They were then diluted 1:100 in LB + Amp + 0.1% arabinose, grown at 25 °C, 30 °C, 37 °C, or 42 °C and harvested at OD_{600} of 0.6. Cell cultures were normalized by OD_{600},

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resuspended in SDS sample buffer, boiled for 10 min, separated by SDS/PAGE, and transferred to nitrocellulose membranes. Western immunoblot was performed with anti-FLAG M2 monoclonal antibody (Sigma), and detection with ECL reagent (GE Healthcare).

Structure Probing Experiments. RNAs were synthesized in vitro by runoff transcription with T7 RNA polymerase from linearized plasmid templates. Plasmids pBO1299 and pBO1845 (linearized with MlsI) were used to generate RNA for enzymatic structure probing. RNA structure probing was carried out at 20 °C, 30 °C, and 37 °C. RNA was 5′-end-labeled as previously described (31). The labeled RNA was then digested with ribonucleases T1 and V1 as previously described (32), and the RNA fragments were separated on denaturing 15% polyacrylamide gels. Alkaline ladders were generated as previously described (31).

CT Assays. *V. cholerae* Δ*toxT* strains KKV2288 and KKV2426 were transformed with plasmids that transcribe *toxT* from the pBAD promoter and translate ToxT from the native or mutant *toxT* 5′ UTRs (pKEK1682, pKEK1683, pKEK1684, and pKEK1685). Cultures were used to measure CT production, as well as *toxT* transcript levels as described below. CT was measured in the supernatant of cultures at OD_{600} of 0.6 grown in LB + Amp + 0.1% arabinose at 25 °C and 37 °C. The ganglioside M_1 ELISA (GM_1-ELISA) was used with polyclonal rabbit serum directed against the purified B subunit of CT as previously described (33).

Quantitative RT-PCR. Total RNA was isolated from *V. cholerae* strains KKV2288 and KKV2426 transformed with plasmids that transcribe *toxT* from the pBAD promoter and translate ToxT from the native or mutant *toxT* 5′ UTRs

(pKEK1682, pKEK1683, pKEK1684, pKEK1685) using TRIzol Max Bacterial RNA Isolation Kit (Ambion). Overnight cultures were inoculated at 1:100 into LB + Amp with 0.1% arabinose. They were grown at 25 °C and 37 °C to OD_{600} of 0.6. The RNA was treated with Turbo DNase (Ambion), and reverse transcription reactions were performed using iScript Reverse Transcription supermix (BioRad); RT-negative controls were included for every RNA sample. cDNA was amplified in the Applied Biosystems ABI 7500 Real-Time PCR System, using SsoAdvanced Universal Syber Green supermix (BioRad), and specific primers for *toxT*, *ctxB*, *tcpB*, and *gyrA* were used. *gyrA* was used as the control. Assays were performed in triplicate. The relative expression values (*R*) were calculated by using the formula $R = 2 - d\mathrm{Ct}\ \text{target} - d\mathrm{Ct}\ \text{endogenous control}$, where Ct is the fractional threshold cycle.

In Vivo Colonization Assay. *V. cholerae* mutant strains were each mixed in a 1:1 ratio with the isogenic wild-type strain and then inoculated intragastrically into 5-d-old CD-1 suckling mice ($\sim 10^6$ mutant: 10^6 wild type). Infected pups were placed in a humidified 30 °C incubator. After 20 h the mice were killed, and their small intestines were isolated and homogenized. The mutant/wild-type ratios were determined by plating dilutions on LB agar containing X-Gal (5-bromo-4-chloro-3-indolyl-ᴅ-galactopyranoside). The competitive index is given as the output ratio of mutant:wild-type divided by the input ratio of mutant:wild-type; Prism 5.0b was used for statistical analyses.

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Supporting Information

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SI Materials and Methods

The plasmid pBAD18-*lacZ*481 (1) was used to create *toxT* 5′ UTR translational fusions. The *toxT* 5′ UTR region from +1 to +71 with respect to the transcription start site (2) was PCR amplified from *Vibrio cholerae* genomic DNA using primers NheI-*toxT* and *toxT*-EcoRI (Table S1), digested with NheI and EcoRI and ligated into pBAD18-*lacZ*481 digested similarly. This created plasmid pBO1296, which transcribes *lacZ* from the pBAD promoter that is translated from the native *toxT* 5′ UTR. *toxT* 5′ UTR mutations U5C and/or U7C were introduced by site-directed mutagenesis (QuikChange, Stratagene; Table S1 provides a complete list of primers) into pBO1296, resulting in pBO1826 (*toxT* 5′ UTR U5C), pBO1829 (*toxT* 5′ UTR U7C), and pKEK1807 (*toxT* 5′ UTR U5C, U7C).

toxT translated from its native 5′ UTR was PCR amplified with oligos WT 5′ UTR Fw NheI and ToxT Rev SphI, then digested with NheI and SphI, and finally ligated into pBAD18-lacZ481 that had been digested similarly to create plasmid pKEK1682, which transcribes *toxT* with its native UTR from the pBAD promoter. *toxT* 5′ UTR mutations U5C and/or U7C were introduced into pKEK1682 by site-directed mutagenesis.

pKEK87 contains *toxT* transcribed from the pBAD promoter and translated from the *araBAD* 5′ UTR (3). *toxT-FLAG* translated from the *toxT* 5′ UTR was created by PCR amplification of pKEK1682, pKEK1683, pKEK1684, and pKEK1685 with oligos ToxT-FLAG Fw and ToxT-FLAG Rev, followed by digestion with DpnI and transformation into DH5α to create pKEK1733 (native 5′ UTR-*toxT*-FLAG), pKEK1734 (U5C UTR *toxT*-FLAG), pKEK1735 (U7C UTR *toxT*-FLAG), and pKEK1736 (U5C, U7C UTR *toxT*-FLAG). The U5C and/or U7C mutations were introduced by site-directed mutagenesis (Stratagene QuikChange) into plasmid pKEK1445 [*toxT* with 1-kb flanking regions in pKAS46 (4)] to create plasmids pKEK1468, pKEK1469, and pKEK1808.

The plasmids used in RNA structure probing experiments were created by PCR amplification of the *toxT* 5′ UTR with primers T7-*toxT* and *toxT*-runoff-SP, and ligation into pUC18 to create plasmid pBO1299. This introduced a T7 promoter sequence upstream and an MlsI site downstream of the *toxT* 5′ UTR. The U5C, U7C mutations were introduced into pBO1299 by site-directed mutagenesis resulting in plasmid pBO1845.

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Fig. S1. The predicted structure of the *toxT* 5′ UTR. Secondary structure derived from Mfold (1), putative fourU thermometer, Shine-Dalgarno (SD) sequence, start codon, and the U5 and U7 residues are indicated.

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Fig. S2. The *toxT* 5′ UTR fourU RNA thermometer facilitates temperature-dependent access to the SD sequence. (*A*) Enzymatic cleavage of 5′ ³²P-end-labeled *toxT* RNA was carried out at 20 °C, 30 °C, and 37 °C. RNA fragments were separated on 15% polyacrylamide. (*A*) RNase T1 (cuts 3′ of single-stranded guanines) was used at the indicated concentrations [0.01 U (+) and 0.1 U (++)]. (*B*) RNase V1 (cuts double-stranded, stacked regions) was used at 0.1 U. Lane L is the alkaline ladder, lane N contains no RNase, and RNA was cleaved with 0.1 U T1 at 42 °C in lane T. WT indicates the native *toxT* 5′ UTR, and U5, 7C indicates the U5C U7C *toxT* 5′ UTR. The SD sequence is labeled, and select nucleotides are noted. These are the full gel images that correspond to Fig. 2.
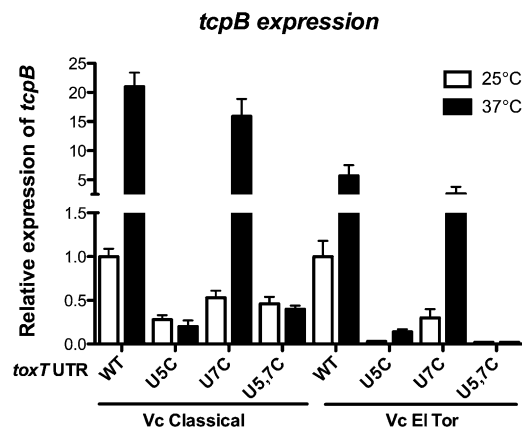
tcpB expression

Fig. S3. The *toxT* 5′ UTR fourU RNA thermometer facilitates temperature-dependent expression of *tcpB*. *V. cholerae* O1 Δ*toxT* strains KKV2288 (Classical) and KKV2426 (El Tor) carrying plasmids pKEK1682 (5′-*toxT* UTR-*toxT*), pKEK1683 (5′-U5C *toxT* UTR-*toxT*), pKEK1685 (5′-U7C *toxT* UTR- *toxT*), or pKEK1686 (5′-U5C U7C *toxT* UTR- *toxT*) were grown at 25 °C and 37 °C in the presence of 0.1% arabinose and harvested at identical cell densities (OD$_{600}$ = 0.6). mRNA abundance for *tcpB* was determined by quantitative RT-PCR (*Materials and Methods*). These results are from the same strains shown in Fig. 4. *tcpB* transcription was higher at 37° than at 25° in strains expressing the native UTR-*toxT*, ($P < 0.001$ classical and $P < 0.01$ El Tor). The U5C and U5C U7C UTRs caused significant decreases in *ctx* transcription ($P < 0.001$ at 37 °C for both classical and El Tor). The U7C UTR had little effect on *ctx* transcription (no significant difference for classical or El Tor).

Table S1. Oligonucleotides used in this study

Name	Sequence
U5C pKAS Fw	GTTATGTAGTTTTATATTAACTTCGACTTTAAGATAACTTTACGTGGATGGCT
U5C pKAS Rev	AGCCATCCACGTAAAGTTATCTTAAAGTCGAAGTTAATATAAAACTACATAAC
U7C pKAS Fw	GTTATGTAGTTTTATATTAACTTCGATTCTAAGATAACTTTACGTGGATGGCTCTCT
U7C pKAS Rev	AGAGAGCCATCCACGTAAAGTTATCTTAGAATCGAAGTTAATATAAAACTACATAAC
U5,7C pKAS Fw	GTAGTTTTATATTAACTTCGACTCTAAGATAACTTTACGTGGATG
U5,7C pKAS Rev	CATCCACGTAAAGTTATCTTAGAGTCGAAGTTAATATAAAACTAC
WT5′UTRFw NheI	GCGCGCTAGCTCGATTTTAAGATAACTTTACGTGGATGGC
U5C Fw NheI	GCGCGCTAGCTCGACTTTAAGATAACTTTACGTGGATGGC
U7C Fw NheI	GCGCGCTAGCTCGATTCTAAGATAACTTTACGTGGATGGC
U5,7C Fw NheI	GCGCGCTAGCTCGACTCTAAGATAACTTTACGTGGATGGC
ToxT Rev SphI	CGCGGCATGCTTATTTTTCTGCAACTCCTGTCAAC
pBAD U5,7C Fw	CCGTTTTTTTGGGCTAGCTCGACTCTAAGATAACTTTACGTGGATGGC
pBAD U5,7C Rev	GCCATCCACGTAAAGTTATCTTAGAGTCGAGCTAGCCCAAAAAAACGG
ToxT-FLAG Fw	GATTACAAGGATGACGATGACAAGTAAAAGCTTGGCTGTTTTGGCGGATGAGAGAAGATT
ToxT-FLAG Rev	TTACTTGTCATCGTCATCCTTGTAATCTTTTTCTGCAACTCCTGTCAACATAAATAAATA
ToxT-FLAG KEK87 Fw	TTTATGTTGACAGGAGTTGCAGAAAAAGATTACAAGGATGACCATGACAAGTAATCTAGAGTCGACCTGCAGGCATGCAAG
ToxT-FLAG KEK87 Rev	CTTGCATGCCTGCAGGTCGACTCTAGATTACTTGTCATCGTCATCCTTGTAATCTTTTTCTGCAACTCCTGTCAACATAAA
ToxT Fw RT	GGCGTTGGGCAGATATTTGT
ToxT Rev RT	TCCTCGAGACTCTAGTTCTTTTTTCA
NheI-*toxT*	TTTGCTAGCTCGATTTTAAGATAAC
toxT-EcoRI	TTTGAATTCAGACATAATGTTAACTCC
toxT-U5C-fw	TCGACTTTAAGATAACTTTACGTGG
toxT-U5C-rv	CCACGTAAAGTTATCTTAAAGTCGA
toxT-U7C-fw	TCGATTCTAAGATAACTTTACGTGG
toxT-U7C-rv	CCACGTAAAGTTATCTTAGAATCGA
toxT-U5,7C-fw	TCGACTCTAAGATAACTTTACGTGG
toxT-U5,7C-rv	CCACGTAAAGTTATCTTAGAGTCGA
T7-*toxT*	GAAATTAATACGACTCACTATAGGGTCGATTTTAAGATAACTTTACG
toxT-runoff-SP	TGGCCATTGCGTTCTACTCTGAAG