Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 9898–9902, December 1990
Genetics

Expression of ToxR, the transcriptional activator of the virulence factors in *Vibrio cholerae*, is modulated by the heat shock response

(cholera toxin/sigma factor/HtpG/HtpR/RpoH)

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Communicated by Boris Magasanik, September 17, 1990

ABSTRACT **The *toxR* gene of *Vibrio cholerae* encodes a transmembrane, DNA-binding protein that positively controls transcription of the genes for cholera toxin, TCP pili, and other proteins important in cholera pathogenesis. Nucleotide sequence analysis of the *toxR* upstream region has revealed that the heat shock gene *htpG*, encoding the bacterial homologue of the eukaryotic Hsp90 protein, was located immediately upstream and was divergently transcribed from *toxR*. Using *lacZ* transcriptional fusions, we have shown that neither *toxR* nor *htpG* expression was regulated by ToxR. However, the growth temperature had a coordinate but reciprocal effect on the expression from both the *toxR* and *htpG* promoters in *V. cholerae*; the decrease of *toxR* expression between 22°C and 37°C was proportional to the increase of *htpG* expression observed within that temperature range. A similar pattern of expression of the *htpG* and *toxR* promoters was observed in the heterologous host *Escherichia coli*, where this regulation was controlled by the level of the *E. coli rpoH* (*htpR*) gene product, σ-32. Consistent with the temperature-regulated expression of the *V. cholerae htpG* promoter in *E. coli*, a sequence similar to the consensus sequence of the *E. coli* heat shock promoters was detected upstream from the *V. cholerae htpG* gene. We propose a model in which the regulation of *toxR* expression by temperature is controlled by the level of σ-32 (RpoH) RNA polymerase.**

The Gram-negative bacterium *Vibrio cholerae* is responsible for the diarrheal disease Asiatic cholera. The most important virulence properties of this organism are enterotoxin production and the ability to adhere to and colonize the small intestine of the host (1). Cholera toxin is a heat-labile enterotoxin composed of one A subunit and five B subunits that are encoded by the *ctxAB* operon (2). Although the signals that regulate the expression of the *ctxAB* operon *in vivo* (i.e., during intraintestinal growth of the bacteria) have not yet been characterized, cholera toxin production can be modulated *in vitro* by changes in osmolarity, pH, and composition of the growth medium, as well as incubation temperature and oxygen tension (3).

The expression of the *ctxAB* operon is under the control of the transcriptional activator encoded by the *toxR* gene (4). In addition to its role in cholera toxin synthesis, the *toxR* gene product is required for the production of the toxin coregulated pilus colonization factor encoded by the *tcp* locus (5) and for the expression of at least eight other genes encoding membrane or exported proteins, some of which are involved in colonization (6). ToxR thus appears to play a central role in the coordinate regulation of *V. cholerae* virulence properties (7).

The *toxR* gene has been cloned from the El Tor strain E7946 in which it is located upstream from and cotranscribed with another gene, *toxS*, the product of which acts in conjunction with ToxR to activate the expression of *ctxAB* (8). The classical strain 569B from which *toxR* was first cloned (4) has a 1.2-kilobase deletion removing the *toxS* coding sequence (8, 9). One hypothesis for the ToxS independence of ToxR activity in strain 569B is that ToxR is expressed from a stronger promoter in this strain, inasmuch as overproduction of ToxR alone indeed leads to activation of the expression of the *ctxAB* promoter in *Escherichia coli* (8–10).

To investigate the regulation of *toxR* expression, we have determined the nucleotide sequence of the DNA fragment located upstream from *toxR*‡ in the El Tor strain E7946 and studied the expression from the *toxR* promoter by use of a *toxR:lacZ* transcriptional fusion. In this report, we present evidence that the *toxR* promoter sequences are identical in strains 569B and E7946 and that *toxR* expression is not autoregulated. The activity of the *toxR* promoter is shown to be modulated by the growth temperature; *toxR* is expressed less at 37°C than at 22°C in *V. cholerae*. This effect, which was also observed in the heterologous host *E. coli*, is probably due to the overlap between the promoters for *toxR* and *htpG*, a heat shock gene that was found upstream and divergently transcribed from *toxR*.

MATERIALS AND METHODS

Bacterial Strains and Growth Media. Derivatives of *V. cholerae* and *E. coli* K-12 were maintained at −70°C in LB medium (11) containing 25% (vol/vol) glycerol or on LB plates. For studies involving isopropyl β-D-thiogalactoside (IPTG) induction of the *ptac:rpoH* fusion, we used NZY broth [5 g of NaCl, 2 g of $MgSO_4 \cdot H_2O$, 5 g of yeast extract, 10 g of NZ amine (ICN) per liter] as growth medium. Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; tetracycline, 15 μg/ml; streptomycin, 100 μg/ml. *E. coli* strain GT869 [*thrB1004*, *pro*, *thi*, *rpsL*, *hsdS*, *lacZΔM15*, F′(*lacZΔM15*, *lacI*^{q}, *traD36*, *proA*$^+$, *proB*$^+$)] (12) was used for cloning experiments, SM10 (*thi*, *thr*, *leu*, *tonA*, *lacY*, *supE*, *recA*::RP4-2Tc::Mu Km) (13) was used to transfer plasmids to *V. cholerae* by conjugation, and MC1061 [*araD139*, Δ(*ara,leu*)*7697*, Δ*lacX74*, *galU*, *galK*, *hsr*$^-$, *hsm*$^+$, *rpsL*, F′(*lacI*^{q})] (14) was used to assay β-galactosidase activity expressed from the recombinant plasmids. *V. cholerae* O395 Sm is a streptomycin-resistant derivative of wild-type O395 (5), and JJM43 is a *toxR* derivative of O395 Sm (5).

β-Galactosidase and Alkaline Phosphatase Assay. β-Galactosidase and alkaline phosphatase activities were assayed as

Abbreviations: ORF, open reading frame; IPTG, isopropyl β-D-thiogalactoside.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M58033).

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detailed by Miller (11) and Peterson and Mekalanos (6), respectively, on cells grown to late exponential phase.

Molecular Cloning Procedures. Plasmid DNA purification, DNA restriction and separation by gel electrophoresis, ligation, and transformations of *E. coli* strains were performed according to the standard methods described by Maniatis *et al.* (15). Nucleotide sequences were determined by the dideoxy chain-termination procedure (16) on double-stranded plasmid DNA. [α-^{35}S]dATP was from Amersham; restriction enzymes, T4 DNA ligase, and the Klenow fragment of *E. coli* DNA polymerase were from New England Biolabs.

Construction of *toxR:lacZ* and *htpG:lacZ* Transcriptional Fusions. The pVJ20 plasmid (8) carries the *toxR–htpG* intergenic region as a 0.6-kilobase-pair-long *Eco*RI–*Eco*RV DNA fragment inserted between the *Eco*RI and the *Sma* I sites of the vector pMLB1010 (17) [i.e., upstream from a *lacZ* reporter gene lacking its own promoter (see Fig. 1)]. The *Eco*RI–*Dra* I DNA fragment of pVJ20, carrying the *toxR:lacZ* transcriptional fusion, was cloned into the filled-in *Bam*HI site of the low-copy-number plasmid pLAFR2 (19) to give rise to pVC201 (Fig. 1). As a control for promoter activity due to the vector part, the *Eco*RI–*Dra* I fragment of pMBL1010, carrying only the *lacZ* reporter gene, was cloned into the filled-in *Bam*HI site of pLAFR2, giving rise to pVC200 (Fig. 1).

An *htpG:lacZ* transcriptional fusion was constructed by inversing the *Eco*RI–*Eco*RV DNA fragment cloned upstream from the *lacZ* reporter gene in pVC201 (*toxR:lacZ*). The *Eco*RI–*Dra* I DNA fragment of pVJ20 was first cloned into the *Hin*cII site of pUC19 (18), yielding pVC100. In this plasmid, pVC100, the *toxR–htpG* intergenic upstream region is flanked by two *Bam*HI sites, located with the pUC19 polylinker and in between the *toxR* and the *lacZ* reporter gene, respectively. Digestion of pVC100 by *Bam*HI and religation allowed the construction of plasmid pVC109 in which the *Bam*HI fragment has been inverted, thus creating a *htpG:lacZ* transcriptional fusion. The *Eco*RI–*Hin*dIII fragment of pVC109, carrying the *htpG:lacZ* fusion, was then cloned into the filled-in *Bam*HI site of pLAFR2 to give rise to pVC202 (Fig. 1). The inserts in pVC200 (*lacZ*), pVC201 (*toxR:lacZ*), and pVC202 (*htpG:lacZ*) are all in the same orientation with respect to the pLAFR2 moiety.

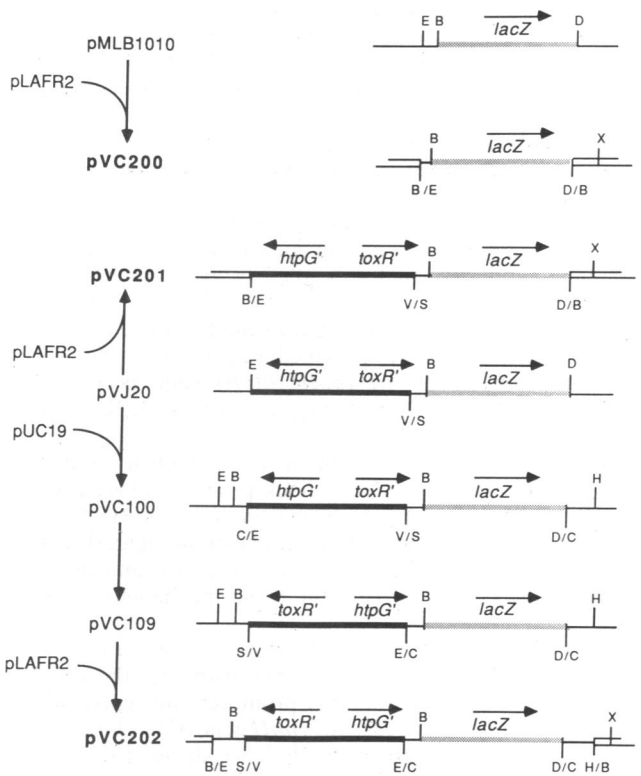

FIG. 1. Construction of the *lacZ*, *toxR:lacZ*, and *htpG:lacZ* reporter plasmids. A schematic map (not drawn to scale) of the inserts carried by the various plasmids is shown along with the position of the relevant restriction sites: B, *Bam*HI; C, *Hin*cII; D, *Dra* I; E, *Eco*RI; H, *Hin*dIII; V, *Eco*RV; S, *Sma* I; X, *Xba* I. A slash (/) between two letters indicates that the sites for the corresponding restriction enzymes have been filled in during the construction (see text). The position and orientation of the 5′ part of the *htpG* and *toxR* genes (black bar) as well as that of the *lacZ* reporter gene (*lacZ*, stippled bar) are indicated by arrows. pMLB1010 (17) and pUC19 (18) are represented by a thin line, and pLAFR2 (19) is represented by an open bar. Construction of plasmid pVJ20 has been described (8).

RESULTS

Nucleotide Sequence of the *toxR* Promoter Region in E7946. The *toxR* gene has been previously cloned from both the classical Inaba strain 569B (4) and from the El Tor strain E7946 (8). The nucleotide sequence of the 0.6-kilobase-pair-long *Eco*RI–*Eco*RV DNA fragment containing the *toxR* upstream region as well as the beginning of the *toxR* gene from strain E7946 was determined from both strands of DNA and is shown in Fig. 2. This sequence differs from the reported sequence of 569B (10) at nucleotides 362 and 427 (Fig. 2), which correspond to nucleotides 1 and 65, respectively, in figure 2 of Miller *et al.* (10). Resequencing this region of 569B indicated that the guanosine nucleotide at position 1 in the previously reported sequence was part of the *Bam*HI site of the vector into which *toxR* was cloned as a DNA fragment partially digested by *Sau*3AI and that the additional guanosine at position 65 was due to a typing error. The sequences from the classical strain 569B and the El Tor strain E7946 are therefore identical over the 200-base-pair-long *toxR* upstream region as well as over the first 11 codons of the *toxR* gene.

The *htpG* Gene Is Located Upstream from *toxR*. Sequence analysis of the *toxR* upstream region indicated the presence of an open reading frame (ORF) oriented opposite to *toxR*, extending from nucleotide 375 to the beginning of the sequenced fragment (Fig. 2). Within this ORF there are two potential translation start sites: the ATG codons at positions 370 and 330, which are preceded by the Shine–Dalgarno-like sequences 5′-GAG-3′ and 5′-GGAG-3′, respectively. The

```
 F  E  A  T  G  S  K  A  I  T  G  L  N  E  I  V  E  D  R  T
GAATTCAGCCGTACCCGATTTAGCAATCGTACCTAAGTTTTCGATCACTTCATCACGGGT  ( 60)

 M  G  I  G  N  D  S  I  T  L  T  N  K  D  K  D  F  S  L  K
CATACCGATCCCGTTATCCGAAATGGTCAACGTATTTTTGTCTTTATCAAAAGAGAGTTT  (120)

 V  G  L  E  A  D  G  Q  Y  L  D  P  H  S  L  A  Q  F  R  L
TACGCCTAATTCAGCGTCGCCCTGATAGAGATCAGGATGAGAAAGCGCTTGAAAACGCAA  (180)

 K  D  V  A  D  S  A  N  S  I  L  E  R  L  F  I  E  K  N  S
CTTATCAACGGCATCGGAGGCGTTAGAAATCAACTCACGCAGGAAAATTTCTTTGTTGGA  (240)

 Y  L  S  H  I  M  L  H  L  L  Q  K  V  E  S  Q  F  G  R  T
GTACAAAGAGTGAATCATCAGGTGCAGCAGTTGTTTAACTTCCGATTGAAAACCACGCGT  (300)

 E  K  N  T  T  A  T  E  S  M  I  A  G  N  V  E  T  E  F  L
TTCTTTATTAGTGGTTGCAGTCTCGCTCATAATCGCTCCGTTTACTTCTGTTTCAAACAA  (360)

 Q  D  M  <-- htpG
TTGATCCATTGAGACTCAATGGAATTACCTTGATGTGCAAGTGAGATATGGACAAAAAAT  (420)

GTAAATTCAAGGTCAAAACTCATAAAAAACACTGTTTTTTGATCGAGATTGGATTATTCTA  (480)

AGTCTGCATTTTTATCAAAGAAGATAAAAAAACCAGTAAAGTCTGAGTGTTGGGACAGGG  (540)

       toxR --> M  F  G  L  G  H  N  S  K  E  I
AGATACTGGGACATTAGATGTTCGGATTAGGACACAACTCAAAAGAGATATC          (592)
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FIG. 2. Nucleotide sequence of the *htpG–toxR* intergenic region. The nucleotide sequence of the *toxR* noncoding strand is shown along with the deduced N-terminal sequences of the ToxR and HtpG proteins. As *htpG* is in opposite orientation to *toxR*, the HtpG sequence should be read from right to left and from bottom to top. The sequence of the −10 and −35 regions of the putative *htpG* promoter (see Fig. 4) are underlined.

translation start was tentatively assigned to the first ATG codon of the ORF. The amino acid sequence deduced from that ORF was compared with the protein sequences in the National Biomedical Research Foundation Library (release 21) using the computer program FASTP (20). This comparison revealed an extensive similarity between the ORF sequence and the sequences of the heat shock proteins of the Hsp90 family. Fig. 3 shows the alignment of the ORF sequence with the N-terminal sequences of Hsp90 from *Saccharomyces cerevisiae* (21), Hsp82 from *Drosophila melanogaster* (22), and HtpG from *E. coli* (23). Of the 105 compared positions between the sequences of *E. coli* HtpG and *V. cholerae* ORF, 91 (86%) are occupied by identical residues. Such an extensive sequence similarity strongly suggests that the ORF located upstream from *toxR* corresponds to the *V. cholerae* equivalent of the *E. coli htpG* gene; this ORF was therefore designated *htpG*.

Neither *toxR* nor *htpG* Expression Is Regulated by ToxR. To investigate the possible involvement of ToxR in the expression of the *toxR* and *htpG* genes, we have constructed reporter plasmids carrying either a *toxR:lacZ* (pVC201) or a *htpG:lacZ* (pVC202) transcriptional fusion (see *Material and Methods*). These plasmids as well as the control vector pVC200 (*lacZ*) were transferred by conjugation to *V. cholerae* classical strains O395 Sm and JJM43, a *toxR* derivative of O395 Sm. β-Galactosidase activity was assayed in the transconjugants inoculated in LB at pH 6.5 or pH 8.4 and grown to late exponential phase at 30°C. These growth conditions are known to modulate the expression of all the ToxR-regulated genes identified so far (6). As shown in Table 1, there was about 10 times and 50 times more β-galactosidase activity expressed from pVC201 (*toxR:lacZ*) and from pVC202 (*htpG:lacZ*), respectively, than from pVC200 (*lacZ*). These results indicated that the *toxR* and *htpG* promoters were both present on the cloned fragment. β-Galactosidase activity expressed from pVC201 (*toxR:lacZ*) was the same after growth at either pH 6.5 or pH 8.4 and was also the same in the $toxR^+$ and $toxR^-$ strains. There is therefore no evidence that ToxR has any effect on the activity of the *toxR* promoter. Similarly, the level of β-galactosidase activity expressed from pVC202 (*htpG:lacZ*) was not affected by the starting pH of the growth medium or by the *toxR* status of the strain, indicating that *htpG* expression is not regulated by ToxR.

Expression of *toxR* and *htpG* Are Both Modulated by the Growth Temperature. The close proximity of the *htpG* and *toxR* genes led us to investigate *toxR* and *htpG* expression at different growth temperatures, temperature being known to regulate expression of the heat shock genes (reviewed in refs. 24 and 25). β-Galactosidase activity was assayed in derivatives of O395 Sm harboring pVC200 (*lacZ*), pVC201 (*toxR:lacZ*), or pVC202 (*htpG:lacZ*) grown to late exponential phase in LB (pH 7.0) at 22, 30, 34, and 37°C.

Whereas β-galactosidase activity expressed from the control plasmid pVC200 (*lacZ*) showed little variation, we observed a 6-fold increase in *htpG* expression between cells grown at 22°C and cells grown at 37°C (Table 2). On the other hand, there was a 5-fold decrease in expression from the *toxR* promoter between cells grown at 22°C and at 37°C. The growth temperature thus appears to have a coordinate and reciprocal effect on the expression from both promoters; the decrease in *toxR* expression is proportional to the increase in *htpG* expression. Induction of *htpG* expression by temperature also supports the conclusion, based on amino acid sequence similarity, that this gene is likely to be the *V. cholerae htpG* gene.

Regulation of the *toxR* and *htpG* Promoters in *E. coli*. In *E. coli*, the heat shock response is mediated by an increase in the level and activity of the *rpoH* (*htpR*) gene product, an alternate σ factor that specifically recognizes the heat shock promoters (26, 27). Upstream of the *V. cholerae htpG* gene, there exists a sequence similar to the consensus sequence derived from the *E. coli* heat shock promoters (see Fig. 4). Together with the induction of the *V. cholerae htpG* promoter at elevated temperatures, these observations suggest that *V. cholerae* is capable of a heat shock response and that this phenomenon is mediated by the same mechanism as in *E. coli*.

We wished to test the hypothesis that an elevation in the level of active RpoH (σ-32) was responsible for the simultaneous derepression of the *htpG* promoter and repression of the *toxR* promoter. Because the *rpoH* gene of *V. cholerae* has not yet been cloned or inactivated, we decided to test the

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HtpG (V.cholerae)  MDQLFETEVNGAIMSETATTNKETRGFQSEVKQLLHLMIHSL ( 42)
                                          ====================
HtpG (E.coli)                        MKGQETRGFQSEVKQLLHLMIHSL ( 24)
                                      - ==  == =- ==- =-= --  --
Hsp90 (S.cerevisiae)                 MASETFEFQAEITQLMSLIINTV ( 23)
                                       === ===== =========
Hsp82 (D.melanogaster)               MPEEAETFAFQAEIAQLMSLIINTF ( 25)

YSNKEIFLRELISNASDAVDKLRFQALSHPDLYQGDAELGVKLSFDKDKNTLTISDNGIGM (107)
================== ===== === ==== ==-== =--====== =========-==
YSNKEIFLRELISNASDAADKLRFRALSNPDLYEGDGELRVRVSFDKDKRTLTISDNGVGM ( 85)
================== ==-=-- == =   = - -= -=--   - - = = = =-==
YSNKEIFLRELISNASDALDKIRYKSLSDPKQLETEPDLFIRITPKPEQKVLEIRDSGIGM ( 84)
======================== ==-==  ==-  -=-=-- =      = = =-====
YSNKEIFLRELISNASDALDKIRYESLTDPSKLDSGKELYIKLIPNKTAGTLTIIDTGIGM ( 86)

TRDEVIENLGTIAKSGTAEF (127)
=======- ========= =
TRDEVIDHLGTIAKSGTKSF (105)
=- =-=  ========== =
TKAELINNLGTIAKSGTKAF (104)
== -=-============
TKSDLVNNLGTIAKSGTKAF (106)
```

FIG. 3. Amino acid sequence comparison of the *V. cholerae* HtpG protein with representatives of the Hsp90 family. The N-terminal sequences of *V. cholerae* (this work, see Fig. 2) and *E. coli* (23) HtpG proteins and those of Hsp90 from *S. cerevisiae* (21) and of Hsp82 from *D. melanogaster* (22) are shown in the one-letter code. Identical (=) and similar (-) residues (I, L, V, and M; R and K; S and T; G and A; F and Y) are indicated between pairs of sequences.

Table 1. β-Galactosidase activity expressed from the *toxR* and *htpG* promoters in $ToxR^+$ and $ToxR^-$ *V. cholerae* strains

Plasmid*	Background†	β-Galactosidase activity,‡ units	
		pH 6.5§	pH 8.4§
pVC200 (*lacZ*)	$toxR^+$	170	230
pVC200 (*lacZ*)	$toxR^-$	210	220
pVC201 (*toxR:lacZ*)	$toxR^+$	2,280	2,080
pVC201 (*toxR:lacZ*)	$toxR^-$	2,060	2,040
pVC202 (*htpG:lacZ*)	$toxR^+$	8,170	10,450
pVC202 (*htpG:lacZ*)	$toxR^-$	14,260	14,860

*See Fig. 1 for the description of pVC200, pVC201, and pVC202.
†O395 Sm and JJM43 were used as the $toxR^+$ and $toxR^-$ strains, respectively.
‡β-Galactosidase activity was assayed according to ref. 11 on *V. cholerae* cells grown to late exponential phase in LB at 30°C. Units are as defined by Miller (11).
§Starting pH of the growth medium.

Table 2. β-Galactosidase activity expressed from the *toxR* and the *htpG* promoters at different growth temperatures

Plasmid*	β-Galactosidase activity,† units			
	22°C	30°C	34°C	37°C
pVC200 (*lacZ*)	130	110	150	250
pVC201 (*toxR:lacZ*)	5350	2240	1,620	1,070
pVC202 (*htpG:lacZ*)	3260	9340	10,900	18,450

*See Fig. 1 for the description of pVC200, pVC201, and pVC202.
†β-Galactosidase activity was assayed according to ref. 11 on *V. cholerae* cells grown to late exponential phase in LB (pH 7.0) at 22, 30, 34 or 37°C. Units are as defined by Miller (11).

```
htpG (E.coli)          CTCTCGCTTGAATTATTCTCCCTTGTCCCCATCTCTCCCA
                         *    ********    *   ******  ***** *
htpG (V.cholerae)      TTTGACCTTGAATTTACATTTTTTGTCCATATCTCACTTG
                       * *  *******           **  ** *
Consensus (E.coli)     T-tC-CcTTGAA - 13/15 nt - CCCCATtTA
```

FIG. 4. Comparison of the *htpG* upstream region with the sequences of heat shock promoters. A sequence located upstream from the *V. cholerae htpG* gene (this work, nucleotides 437 to 398 in Fig. 2) has been aligned with the sequence of the *E. coli htpG* promoter (23) and with the consensus sequence derived from the *E. coli* heat shock promoters (24). Identical nucleotides are indicated by stars between the sequences. nt, nucleotides.

ability of the *E. coli rpoH* gene product to mediate this regulation. In these experiments, we used an *E. coli lacZ* strain carrying either pVC201 (*toxR:lacZ*) or pVC202 (*htpG:lacZ*) and an IPTG-inducible *rpoH* construct encoded by plasmid pDS2 (34). As shown in Table 3, the expression from the *toxR* and *htpG* promoters was reduced and increased, respectively, between *E. coli* cells grown at 22°C and 37°C. This temperature regulation was enhanced by the presence of pDS2 (p*tac:rpoH*), even without IPTG induction of the cloned *rpoH* gene. The addition of IPTG was found to further repress the *toxR* promoter, and this effect was dependent on pDS2 at both 22°C and 37°C, in that the control plasmid vector pKK233-3 did not mediate this regulation. This decrease in the *toxR* promoter activity was matched by a roughly proportional increase in the expression from the *htpG* promoter (see pVC202, Table 3).

Effect of Temperature on the Expression of ToxR-Regulated Genes. To test the effect of temperature on the expression of the ToxR-regulated genes, alkaline phosphatase activity was assayed in four representative strains with Tn*phoA* (28) insertions in *ctxA*, *tcpA*, *acfA*, and *tagA*, grown in LB (pH 6.5) at either 30°C or 37°C. The expression of each of these Tn*phoA* fusions was reduced between 12- and 32-fold by growth at 37°C (data not shown).

DISCUSSION

In the El Tor strain E7946, activation of the cholera toxin promoter requires the products of both the *toxR* and the *toxS* genes, which are organized in an operon (ref. 8; V. DiRita and J.J.M., unpublished results). This genetic organization is general for all strains studied so far, except for the hypertoxinogenic strain 569B, which has a 1.2-kilobase deletion removing the *toxS* coding sequence (9). Here we have reported the nucleotide sequence of the *toxR* upstream region in strain E7946 and shown that it is identical to the *toxR* upstream region in strain 569B over the 200-base-pair-long region located in between the *toxR* and *htpG* coding sequences. This suggests that the secondary mutation(s) selected through animal passages that gave rise to the hypertoxinogenic strain 569B might not affect *toxR* expression but rather the expression or the structure of another gene(s) also involved in the regulation of toxin synthesis. A potential candidate for this other gene(s) is the *toxT* locus, which has recenty been cloned from 569B through its ability to activate transcription of the *ctx* promoter in *E. coli* (V. DiRita, C.P., G. Jander, and J.J.M., unpublished results).

Table 3. Regulation of the *toxR* and *htpG* promoters by temperature and RpoH in *E. coli*

Reporter plasmid	Activator plasmid*	IPTG†	β-Galactosidase activity,‡ units 22°C	37°C
pVC201 (*toxR:lacZ*)	pKK233-3 (p*tac*)	−	4,080	2,100
pVC201 (*toxR:lacZ*)	pKK233-3 (p*tac*)	+	4,460	2,020
pVC201 (*toxR:lacZ*)	pDS2 (p*tac:rpoH*)	−	2,890	670
pVC201 (*toxR:lacZ*)	pDS2 (p*tac:rpoH*)	+	900	490
pVC202 (*htpG:lacZ*)	pKK233-3 (p*tac*)	−	6,890	8,900
pVC202 (*htpG:lacZ*)	pKK233-3 (p*tac*)	+	9,440	10,760
pVC202 (*htpG:lacZ*)	pDS2 (p*tac:rpoH*)	−	11,120	19,170
pVC202 (*htpG:lacZ*)	pDS2 (p*tac:rpoH*)	+	17,310	24,490

All plasmids were introduced into the *E. coli lacZ* strain MC1061 (F′ *lacI*^{q}), and the transformants were grown to late exponential phase in NZY broth at 22°C or 37°C. See Fig. 1 for the description of pVC201 and pVC202.

*pDS2 contains the *E. coli rpoH* gene cloned under the control of the *tac* promoter (p*tac*) of the vector pKK233-3.

†Bacteria were grown in either the presence (+) or absence (−) of 1 mM IPTG.

‡Units of β-galactosidase activity are as defined by Miller (11).

The proportionality between the decrease of *toxR* expression and the increase of *htpG* expression suggests that there is a relationship between the two phenomena. Examination of the *htpG* upstream region revealed a sequence similar to the consensus sequence derived from the *E. coli* heat shock promoters, an indication that the *V. cholerae* heat shock genes, like those of *E. coli*, should be expressed from promoters recognized by a σ-32 (Rpo-H) RNA polymerase (26, 27). This was confirmed by looking in *E. coli* at the expression of the *V. cholerae htpG* promoter, which was indeed induced by either elevating the temperature or by increasing the level of the *E. coli rpoH* gene product at a given temperature through IPTG induction of a p*tac:rpoH* construct. The decrease of *toxR* expression at elevated temperatures in both *V. cholerae* and *E. coli* suggests that the *toxR* promoter is recognized by a σ factor different from RpoH, perhaps σ-70 in *E. coli* and its equivalent in *V. cholerae*. Thus, the *toxR* and *htpG* promoters are probably recognized by two different types of RNA polymerases, one with σ-70 for *toxR* and the other with σ-32 for *htpG*. If, due to the proximity of the two divergent promoters, the access to the *htpG–toxR* intergenic region is restricted to only one RNA polymerase at a time, then the ratio of *toxR* vs. *htpG* expression might be controlled by the concentration of σ-32 RNA polymerase in the cell. This indeed appears to be the case in *E. coli* since artificially elevated levels of RpoH are sufficient to repress the *toxR* promoter and induce the *htpG* promoter. Previously, evidence that a promoter can be controlled by restricted access and competition between two different forms of RNA polymerase (i.e., σ-54 and σ-70) has been reported by Reitzer *et al.* (30). Because the level of both σ-32 and σ-70 is known to be increased at elevated growth temperatures (24), the switch between expression of *toxR* and *htpG* observed upon elevating the growth temperature is probably a consequence of the absolute level of σ-32 RNA polymerase rather than a change in the ratio of σ-70 and σ-32 RNA polymerases. In this model, the control mechanism of *toxR* expression by temperature appears both simple and sophisticated: sophis-

ticated in the sense that it is governed by the heat shock response, which is a universal and tightly controlled phenomenon, and simple as it does not apparently require any specific component for the regulation of *toxR* except the close proximity of the *htpG* and *toxR* promoters. Proof that this is the mechanism of control awaits the construction of *htpG* promoter down mutations, which should simultaneously lead to derepression of the *toxR* promoter independent of temperature.

The decreased expression of *toxR* at 37°C seems paradoxical for the gene encoding a transcriptional activator of the virulence factors of a human pathogen. If this regulation is a consequence of a heat shock response, then it is probably not restricted to *in vitro* growth conditions and should occur during the intraintestinal growth of the bacteria, as there is no environmental factor known to antagonize the heat shock response. There is little doubt that cholera toxin is synthesized during *V. cholerae* infections (reviewed in ref. 31) and that this synthesis is under the control of ToxR. Demonstration that deletion mutations in *toxR* eliminate intestinal colonization in animals (5) and in human volunteers (32) provides ample evidence for the importance of the ToxR regulon in cholera pathogenesis. It is not known whether the level of *toxR* expression has a direct effect on the regulation of any ToxR-regulated gene during intraintestinal growth. That ToxR-regulated genes are poorly expressed *in vitro* at 37°C is consistent with the decreased *toxR* expression but should be considered cautiously inasmuch as temperature might also inhibit ToxR activity in the absence of an activating environmental effector or signal.

Our study has dealt with the steady-state level of expression of *htpG* and *toxR*, in line with the known temperature of the bacterial growth during pathogenesis. It is likely that upon a temperature shift, such as the one encountered by the bacteria when entering the human body, the expression of the heat shock genes is going to be strongly induced. This heat shock response might help *V. cholerae* to survive adverse environmental conditions, such as exposure to the low pH of the stomach, bile salts, or perhaps anaerobiosis and starvation. Indeed, heat shock proteins have been proposed to play a role in the recovery from adverse environmental conditions by facilitating the breakdown of denatured proteins or helping in their renaturation (25). It is also well known that the pattern of gene derepression seen in heat shock overlaps the patterns seen after several different types of stress (e.g., anoxia, starvation, and exposure to organic solvents and other noxious compounds) (24, 25). According to our model, upon induction of *htpG* expression, the expression of *toxR* should be proportionally decreased and perhaps lead to reduced expression of the ToxR-activated genes (*tag* genes). This transient lag in the expression of the *tag* genes may avoid synthesis of cholera toxin and TCP pili too early in the pathogenic process (i.e., prior to mucus penetration and adherence of vibrios to the epithelial cells). Likewise, decreased ToxR synthesis may lead to increased expression of ToxR-repressed genes (*trg* genes) such as *ompT* (5) and some genes involved in motility and chemotaxis (C. Gardel and J.J.M., unpublished results), which might directly contribute to the success of the vibrios in the early stage of the infection cycle. Once *V. cholerae* reaches the appropriate anatomical site, signal transduction by ToxR (i.e., the sensing of environmental signals presumably at the mucosal surface) would lead to expression of ToxR-activated genes even under conditions of relatively low levels of *toxR* expression. In this regard, it is of interest to note that the *toxS* gene product has been found to enhance ToxR activity under conditions of low *toxR* transcription (33). Thus, a complex regulatory cascade involving at least two σ factors (σ-32 and σ-70), ToxR, ToxS, and other transcriptional factors, such as ToxT, no doubt acts at various stages of the *V. cholerae* infection cycle to fine tune the bacterium's response to the host environment.

We thank V. DiRita and K. Killeen for helpful discussions and W. Walter and C. Gross for the kind gift of the pDS2 plasmid. This work was supported by Public Health Service Grants AI-18045 and AI-26289. C.P. was a Research Fellow from the Institut Pasteur.

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