JOURNAL OF BACTERIOLOGY, May 1994, p. 3085–3088
0021-9193/94/$04.00+0

Vol. 176, No. 10

The Light Organ Symbiont *Vibrio fischeri* Possesses a Homolog of the *Vibrio cholerae* Transmembrane Transcriptional Activator ToxR

KARL A. REICH[1] AND GARY K. SCHOOLNIK[1,2]*

Department of Microbiology and Immunology[1] and Howard Hughes Medical Institute,[2] Stanford University Medical School, Stanford, California 94305-5428

Received 10 February 1994/Accepted 14 March 1994

A cross-hybridizing DNA fragment to *Vibrio cholerae toxR* was cloned from the nonpathogenic light organ symbiont *Vibrio fischeri*, and three proteins homologous to *V. cholerae* ToxR, ToxS, and HtpG were deduced from its DNA sequence. *V. fischeri* ToxR was found to activate a *V. cholerae* ToxR-regulated promoter, and an antiserum raised against the amino-terminal domain of *V. cholerae* ToxR cross-reacts *V. fischeri* ToxR.

While cholera epidemics are well documented phenomena, the habitat of the organism between outbreaks of human disease is controversial, though the ability of *Vibrio cholerae* to survive in estuarine habitats in association with marine invertebrates has been documented (2). The capacity to persist in marine environments has also been well established for several nonpathogenic *Vibrio* species, especially those that are luminescent and colonize the light organ, a highly evolved and complex structure of certain species of fish and squid (7, 13). Like *V. cholerae* which shows specificity for the human intestine, the luminous bacteria that colonize the light organ exhibit a narrow host range and a highly specific tissue tropism. Moreover, like *V. cholerae*, many light organ symbionts live in at least two very different environments, either as free-living forms in seawater or as forms closely associated with their marine hosts, adjacent to the epithelium of the light organ in a manner that resembles the juxtaposition of *V. cholerae* and the mucous membranes of the human intestine (8).

These similarities led us to examine the hypothesis that genes that are required for the infection of the human intestine by *V. cholerae* are present as structural and functional homologs in nonpathogenic marine *Vibrio* species. One such protein, ToxR, regulates cholera toxin production as a function of environmental variables (4). Here we describe the cloning and initial characterization of a ToxR homolog from the nonpathogenic light organ symbiont *Vibrio fischeri*. The data presented below raise the possibility that homologous regulatory sequences may be required for pathogenicity by one species and for symbiosis by another.

Amino acid sequence comparison of *V. fischeri*, *V. cholerae*, and *Vibrio parahaemolyticus* ToxR and ToxS homologs. A size-selected library of *V. fischeri* genomic DNA was constructed in a multicopy vector (pBS KSII$^+$), and recombinant clones were isolated in groups of 100. The plasmid DNA from these pools was isolated, digested to release the cloned inserts, and screened by Southern hybridization at reduced stringency (hybridized in 10% formamide–2× SSC [1× SSC is 0.15 MNacl plus 0.015 M sodium citrate] at 42°C and washed sequentially to 0.125× SSC at 42°C) with a PCR-generated *V. cholerae toxR* probe. Positive pools were subdivided and rescreened until a positive colony was isolated. This strategy yielded a 4.7-kbp *Xba*I fragment from *V. fischeri* MJ-A1 (Table 1) that cross-hybridized with *V. cholerae toxR*. The DNA sequence of the hybridizing 4.7-kbp *Xba*I fragment was determined, and the analysis of this sequence led to the identification of three open reading frames homologous to three previously described genes from *V. cholerae*: *toxR*, *toxS*, and *htpG*. The amino acid sequence alignments of *V. fischeri* ToxR and ToxS with *V. cholerae* ToxR and ToxS and with the recently published (6) sequences of *V. parahaemolyticus* ToxR and ToxS are shown in Fig. 1. Also shown in Fig. 1 is a comparison of the amino acid sequence of *V. fischeri* HtpG with a previously published partial sequence of *V. cholerae* HtpG (12).

Inspection of these sequence alignments reveals that the overall amino acid identity between *V. fischeri* ToxR and *V. cholerae* ToxR is 43%. In addition, both proteins apparently lack an N-terminal signal sequence and a single stretch of highly hydrophobic amino acids unequally divides the protein into an (presumed) amino-terminal cytoplasmic domain and a carboxy-terminal periplasmic domain. However, the homologous residues are not evenly distributed along the sequence but are present in two large blocks: one in the amino-terminal domain and the other in the carboxy-terminal domain.

The region between the above noted amino- and carboxy-terminal sequences is only 30% similar and consists of the final half of the N-terminal domain, the hydrophobic sequence which may constitute a transmembrane domain, and the beginning of the C-terminal domain (amino acids [aa] 110 to 225). However, within this less conserved region, there is a striking conservation of the number and spacing of proline residues (Fig. 1A). In comparing *V. fischeri* ToxR with *V. parahaemolyticus* ToxR, the same general structural plan is evident, including the presence of two major homologous blocks that are separated by a putative transmembrane domain and by a region with little amino acid identity (Fig. 1A).

The overall amino acid identity between *V. fischeri* ToxS and *V. cholerae* ToxS is 42%. Although less is known about the functional domains of the ToxS protein of *V. cholerae* (3), a putative amino-terminal, hydrophobic, membrane anchor region is retained in *V. fischeri* ToxS (Fig. 1B).

The third open reading frame of the 4.7-kbp *Xba*I fragment predicted an amino acid sequence that clearly aligned with the HtpG family of heat shock proteins (Fig. 1C). Although only a partial sequence of *V. cholerae* HptG has been published (12), the *V. fischeri* HtpG and *V. cholerae* HtpG sequences are

* Corresponding author. Mailing address: Stanford University Hospital, Beckman Center, Room B241, Stanford, CA 94305-5428. Phone: (415) 723-8158. Fax: (415) 723-1399.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristic	Source or reference
Strains		
V. fischeri MJ-A1	Light organ symbiont	P. Dunlap (5)
V. cholerae 0395	Classical cholera strain	Laboratory collection
E. coli		
DH5αF′	*recA1*, restriction negative	Laboratory collection
VM2	*E. coli*/*ctx*::*lacZ* promoter fusion	9
Plasmids		
pBS KSII$^+$	Cloning vector	Stratagene
p*Trc*99A	Expression vector, IPTG-inducible *trc* promoter	Pharmacia
p*Trc*99A/Vf-*toxR*	*V. fischeri* ToxR cloned into *Eco*RI–*Pst*I sites	This study
p*Trc*99A/Vc-*toxR*	*V. cholerae* ToxR cloned into *Nco*I–*Pst*I sites	This study
p*Trc*99A/Vc-*toxR*(Δ1–13)	*V. cholerae* ToxR aa 13 to 281 cloned into *Nco*–*Pst*I sites	This study

essentially identical; they have diverged much less than the amino acid sequences of the ToxR and ToxS homologs of *V. fischeri* and *V. cholerae*.

Genomic organization of *V. fischeri toxR*, *toxS*, and *htpG*. Analysis of the open reading frames within the 4.7-kbp *Xba*I fragment showed that *V. fischeri toxR* and *toxS* are both transcribed in the same direction and in the opposite orientation to that of *V. fischeri htpG*. This genomic organization is conserved in *V. cholerae* (3, 10, 12). However, unlike the *V. cholerae toxRS* system, the 3′ end of the coding sequence of *V. fischeri toxR* overlaps the 5′ start of *toxS* by 79 nucleotides, and the reading frames are offset by one nucleotide. The intergenic distance between the divergently transcribed *V. fischeri htpG* and *toxR* genes is 230 bp compared with 189 for *V. cholerae htpG* and *toxR*.

In order to confirm that the deduced amino acid sequence of *V. fischeri toxR* does in fact code for a protein product, lysates of *V. cholerae* and *V. fischeri* were analyzed by Western blot (immunoblot) (Fig. 2). The antiserum that we used was raised against a purified recombinant 193-amino-acid N-terminal fragment of *V. cholerae* ToxR [ToxR(N)]. Although this antiserum was elicited with *V. cholerae* ToxR(N), it also reacted with native *V. fischeri* ToxR, demonstrating that *V. fischeri* ToxR and *V. cholerae* ToxR share common antigenic determinants. The molecular weights of *V. fischeri* ToxR and *V. cholerae* ToxR measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) correspond well with the molecular weights calculated from the deduced amino acid sequences (35,689 versus 32,524).

Activation of the cholera toxin promoter by *V. fischeri* ToxR. In order to adduce further evidence that *V. fischeri* ToxR might be functionally as well as structurally related to *V. cholerae* ToxR, we tested whether *V. fischeri* ToxR could activate the cholera toxin promoter by using the promoter fusion strain VM2 (Table 1) (9). This *Escherichia coli* strain harbors a recombinant lambda phage containing the upstream regulatory region of the cholera toxin operon (*ctx*) and the first 23 amino acids of the A subunit of cholera toxin fused to the coding region of β-galactosidase (*lacZ*). Recognition and activation of the *ctx* promoter were then determined by assaying for β-galactosidase activity. In a previous study by Miller and Mekalanos (9), the presence of *V. cholerae* ToxR in VM2 led to the induction of the *ctx* promoter and an increase in β-galactosidase activity. Here we used VM2 to perform an analogous experiment to test whether *V. fischeri toxR*, placed under the control of an inducible promoter, could act as a functional homolog of *V. cholerae toxR*.

The use of PCR primers permitted us to precisely clone *toxR* from *V. cholerae* and *V. fischeri* with an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter in the commercially available expression vector p*Trc*99A (Table 1). These constructs were individually transformed into strain VM2, and the ability of the resulting clones (p*Trc*99A/Vf-*toxR* and p*Trc*99A/Vc-*toxR* for *V. fischeri* and *V. cholerae*, respectively) to activate the *ctx*::*lacZ* promoter fusion was evaluated both before and after IPTG induction. Control strains included VM2 alone and VM2 transformed with vector p*Trc*99A alone. This experiment (Fig. 3) demonstrated that activation of *ctx*::*lacZ* by *V. cholerae* ToxR yields approximately 600 U of β-galactosidase activity 3.5 h after the addition of IPTG. *V. fischeri* ToxR also activates the *ctx*::*lacZ* fusion but at a lower level, yielding 200 U of β-galactosidase activity. In contrast, the negative control strains and the two strains containing either *V. cholerae* ToxR or *V. fischeri* ToxR prior to the addition of IPTG produced less than 45 U of β-galactosidase activity. Thus, although *V. fischeri* ToxR was less active than its *V. cholerae* ToxR homolog, both proteins activated the cholera toxin promoter and therefore are functionally similar in this respect.

Identification of the initiator methionine start site of *V. cholerae* ToxR. In a prior section of this report (Fig. 1), comparison of the predicted *V. cholerae* ToxR and *V. fischeri* ToxR amino acid sequences was discussed. In addition to the identification of homologous regions reported, the alignments depicted in Fig. 1 show that the initiating methionine residues for both *V. fischeri* ToxR and *V. parahaemolyticus* ToxR coincide with Met-13 of *V. cholerae* ToxR, prompting us and other investigators to question which methionine residue might be the correct translation start site for *V. cholerae* ToxR (6). To ascertain whether use of the alternate start site would lead to an active *V. cholerae* ToxR variant, the VM2 host *ctx*::*lacZ* fusion assay system was employed to test the activity of a mutant *V. cholerae* ToxR lacking the first 13 N-terminal amino acids. For this purpose, a new PCR oligonucleotide primer that would allow the direct cloning of *V. cholerae* ToxR(Δ1–13) into p*Trc*99A was designed, and this construct was transformed into strain VM2. Lysates from the VM2 strain harboring cloned *V. cholerae* ToxR(Δ1–13) were analyzed by Western blot with antisera made with *V. cholerae* ToxR(N). Although an immunologically reactive *V. cholerae* ToxR variant was synthesized (data not shown), this deletion of the recombinant *V. cholerae* ToxR could not activate the *ctx*::*lacZ* promoter fusion, yielding only 50 to 60 U of β-galactosidase (Fig. 2). We conclude from these experiments that *V. cholerae* ToxR requires its N-terminal extension for full activity and that the previously proposed protein translation start site (11) is the correct one.

On the basis of the constellation of similar structural, functional, and genetic features, we conclude that *V. fischeri* ToxR is a member of the ToxR family of regulatory proteins

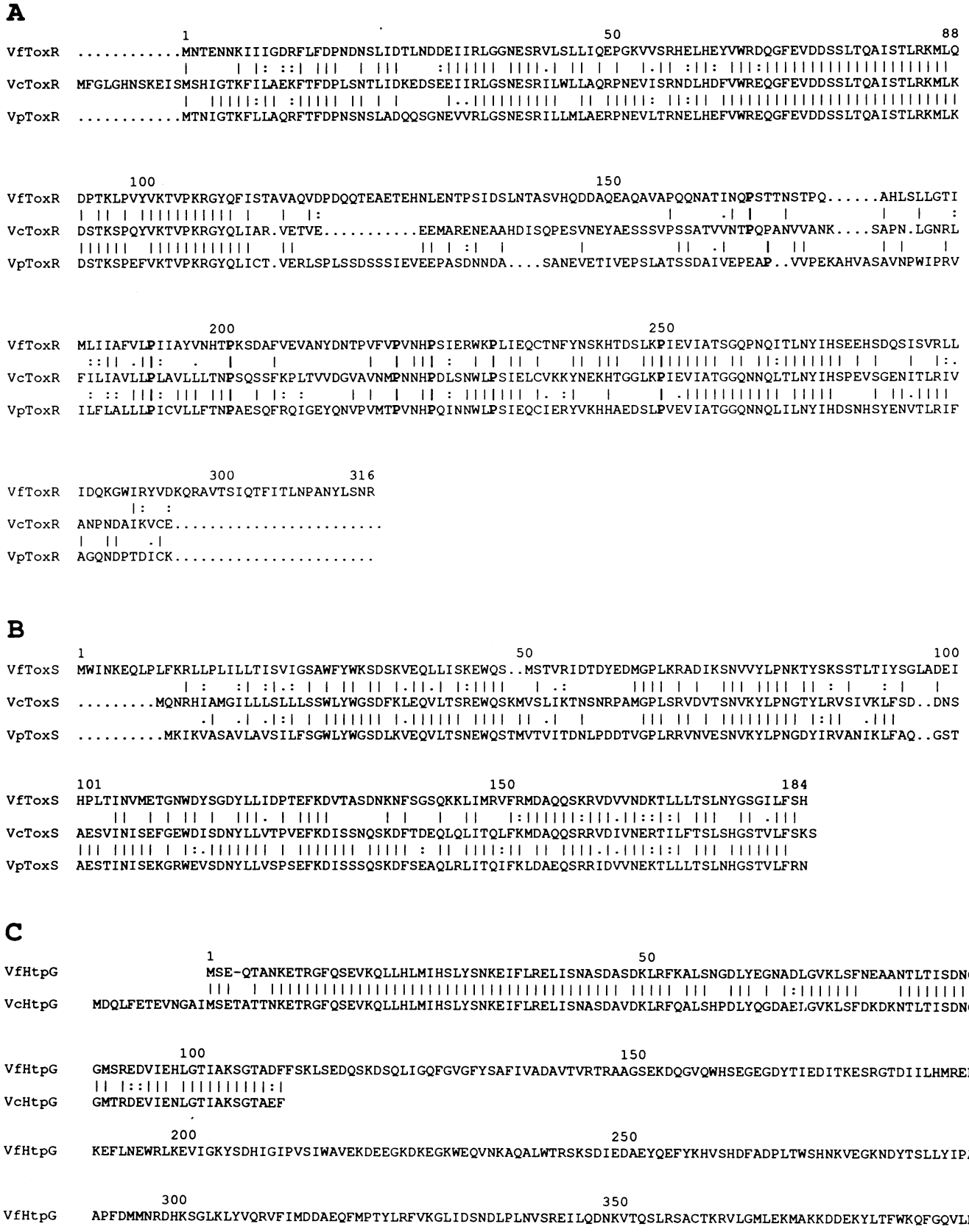

FIG. 1. Amino acid alignment of ToxR (A) and ToxS (B) homologs from *V. fischeri*, *V. cholerae*, and *V. parahaemolyticus* and HtpG (C) homologs from *V. fischeri* and *V. cholerae*. Amino acid identity is shown by vertical lines (|), conservative substitutions (E and D, R and K, I and L) are shown by colons (:), and similar amino acids are shown by dots (.). Conserved prolines in the ToxR sequences are shown in boldface. Structural features of ToxR include the amino-terminal cytoplasmic domain (aa 1 to ~176), putative transmembrane spanning domain (aa 177 to ~201), and periplasmic carboxy-terminal domain (aa 202 to 316). Vf, *V. fischeri*; Vc, *V. cholerae*; Vp, *V. parahaemolyticus*.

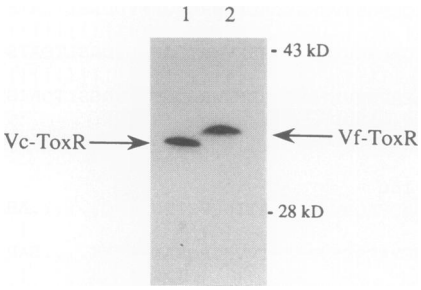

FIG. 2. Western blot probed with anti-ToxR affinity-purified antiserum. SDS–15% PAGE gel was transferred to Immobilon P. Lanes: 1, *V. cholerae* lysate; 2, *V. fischeri* lysate. Molecular masses (in kilodaltons) are indicated on the right. Vc-ToxR, *V. cholerae* ToxR; Vf-ToxR, *V. fischeri* ToxR.

and that *V. fischeri* ToxR and *V. cholerae* ToxR are true homologs. Given the presence of a ToxR-like protein in *V. fischeri*, it is perhaps not surprising that a gene homologous to *V. cholerae toxS* might also be present. What was surprising is that the arrangement of *toxR*, *toxS*, and *htpG* in *V. fischeri* is identical to their genomic organization in *V. cholerae* (3, 12). The conservation of this motif is additional evidence that this genomic organization might be biologically significant. The arrangement of *toxR* and *toxS* is also found to be similar in *V. parahaemolyticus* (6) in which *toxR* and *toxS* are adjacent and transcribed in the same direction.

Our work on ToxR was initiated because of the well-known discrepancy between the conditions for achieving maximal activation of ToxR-regulated promoters in vitro (pH 6, 30°C) and in the human intestinal milieu (pH 7.8, 38°C). We therefore speculated that ToxR might regulate functions involved in the survival of *V. cholerae* in the marine environment, and this led us to hypothesize that evolutionary precursors or homologs of proteins and functions required by pathogenic *V. cholerae* might be found in nonpathogenic *Vibrio* species whose biological niche (the light organ symbionts) predates *Homo sapiens*. We tested this idea by screening these strains for *V. cholerae toxR* homologous sequences by using low stringency Southern hybridization and a *V. cholerae toxR* probe.

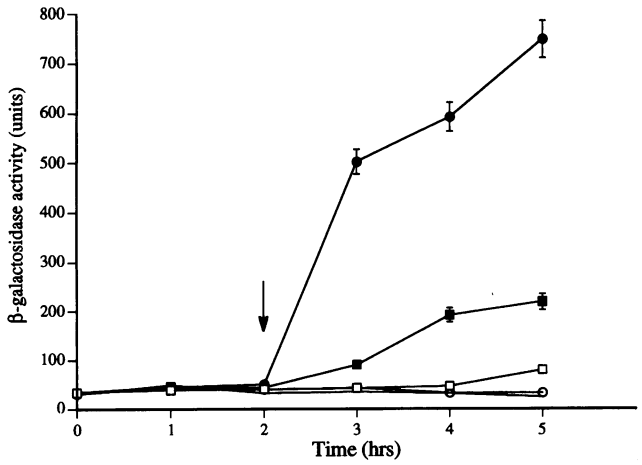

FIG. 3. Activation of *ctx* promoter by *V. fischeri* ToxR. β-Galactosidase activity from strain VM2 carrying p*Trc*99A/Vc-*toxR* (●), p*Trc*99A/Vf-*toxR* (■), p*Trc*99A with no insert (○), or p*Trc*99A/Vc-ToxR(Δ1-13) (□) or from strain VM2 alone (⊙) was measured at the indicated times. Cultures were induced with 1 mM IPTG (addition indicated by vertical arrow). Error bars that were smaller than the size of the symbols were omitted.

Both *V. cholerae* ToxR and *V. parahaemolyticus* ToxR apparently regulate genes involved directly and indirectly in virulence, including exotoxin production and functions involved in colonization (4). *V. fischeri*, a luminous, nonpathogenic marine organism, colonizes the light organ of several fish and squid species (5, 13). Bacterial determinants involved in attachment, colonization, and persistence in the light organ have not yet been identified, although an experimental system has been developed to begin to address these questions (13). We hypothesize that *V. fischeri* ToxR will be required for successful colonization of the light organ by *V. fischeri*. We have identified, and are characterizing, an ADP-ribosyltransferase from *V. fischeri* and are constructing *V. fischeri* ToxR null strains. The rigorous test of the in vivo function of *V. fischeri* ToxR will require characterizing complemented *V. fischeri* ToxR null strains for their capacity to function as symbionts and a more complete description of the ADP-ribosyltransferase and its in vivo target.

Nucleotide sequence accession number. The complete nucleotide sequence of *V. fischeri htpG toxR toxS* has been submitted to GenBank and EMBL and given accession no. L29053.

We thank Paul Dunlap and Ed Ruby for their generous donation of *V. fischeri* strains.

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1991. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
2. **Colwell, R. R., J. Kaper, and S. W. Joseph.** 1977. *Vibrio cholerae*, *Vibrio parahaemolyticus*, and other vibrios: occurrence and distribution in Chesapeake Bay. Science **198**:394–396.
3. **DiRita, V. J., and J. J. Mekalanos.** 1991. Periplasmic interaction between two membrane regulatory proteins, ToxR and ToxS, results in signal transduction and transcriptional activation. Cell **64**:29–37.
4. **DiRita, V. J., C. Parsot, G. Jander, and J. J. Mekalanos.** 1991. Regulatory cascade controls virulence in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **88**:5403–5407.
5. **Fukasawa, S., P. Dunlap, Y. Benno, and T. Mitsuoka.** 1984. Growth and luminescence of luminous bacteria on modified Haneda's medium. Microbiol. Immunol. **28**:117–121.
6. **Lin, Z., K. Kumagai, K. Baba, J. J. Mekalanos, and M. Nishibuchi.** 1993. *Vibrio parahaemolyticus* has a homolog of the *Vibrio cholerae toxRS* operon that mediates environmentally induced regulation of the thermostable direct hemolysin gene. J. Bacteriol. **175**:3844–3855.
7. **McFall, N. M., and E. G. Ruby.** 1991. Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. Science **254**:1491–1494.
8. **McFall-Ngai, M., and M. K. Montgomery.** 1990. The anatomy and morphology of the adult bacterial light organ of *Euprymna scolopes* Berry (Cepahalopoda:Sepiolidae). Biol. Bull. **179**:332–339.
9. **Miller, V. L., and J. J. Mekalanos.** 1984. Synthesis of cholera toxin is positively regulated at the transcriptional level by toxR. Proc. Natl. Acad. Sci. USA **81**:3471–3475.
10. **Miller, V. L., and J. J. Mekalanos.** 1985. Genetic analysis of the cholera toxin-positive regulatory gene *toxR*. J. Bacteriol. **163**:580–585.
11. **Miller, V. L., R. K. Taylor, and J. J. Mekalanos.** 1987. Cholera toxin transcriptional activator ToxR is a transmembrane DNA binding protein. Cell **48**:271–279.
12. **Parsot, C., and J. J. Mekalanos.** 1990. Expression of ToxR, the transcriptional activator of the virulence factors in *Vibrio cholerae*, is modulated by the heat shock response. Proc. Natl. Acad. Sci. USA **87**:9898–9902.
13. **Ruby, E. G., and N. M. McFall.** 1992. A squid that glows in the night: development of an animal-bacterial mutualism. J. Bacteriol. **174**:4865–4870.