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A Region of the Transmembrane Regulatory Protein ToxR That Tethers the Transcriptional Activation Domain to the Cytoplasmic Membrane Displays Wide Divergence among *Vibrio* Species

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The virulence regulatory protein ToxR of *Vibrio cholerae* is unique in that it contains a cytoplasmic DNA-binding–transcriptional activation domain, a transmembrane domain, and a periplasmic domain. Although ToxR and other transmembrane transcriptional activators have been discovered in other bacteria, little is known about their mechanism of activation. Utilizing degenerate oligonucleotides and PCR, we have amplified internal *toxR* gene sequences from seven *Vibrio* and *Photobacterium* species and subspecies, demonstrating that *toxR* is an ancestral gene of the family *Vibrionaceae*. Sequence alignment of all available ToxR amino acid sequences revealed a region between the transcriptional activation and transmembrane domains that displays wide divergence among *Vibrio* species. We hypothesize that this region merely tethers the transcriptional activation domain to the cytoplasmic membrane and thus can tolerate wide divergence and multiple insertions and deletions. The divergence in the tether region at the nucleotide level may provide a useful tool for the distinction of *Vibrio* and *Photobacterium* species.

The bacterium *Vibrio cholerae* expresses virulence factors that allow it to colonize the human intestine and cause the disease cholera. The transmembrane transcriptional activator protein ToxR of *V. cholerae* is required for coordinate expression of virulence factors, including cholera toxin and the toxin-coregulated pilus (13, 17). When discovered, ToxR was unique in that it was the first example of a transcriptional activating protein that also contained a transmembrane segment and periplasmic domain in addition to the cytoplasmic DNA-binding domain. It was hypothesized that the periplasmic domain allowed ToxR to directly sense the extracellular environment and activate the transcription of virulence genes only under inducing conditions. However, since the discovery of ToxR, it has become clear that induction of virulence factor expression in *V. cholerae* is much more complicated. ToxR requires another transmembrane transcriptional activator, TcpP, in order to synergistically activate transcription of *toxT*, which encodes yet a third transcriptional activator that is entirely cytoplasmically located (5, 7). ToxT is the direct transcriptional activator of the *ctx* and *tcp* genes, which encode the cholera toxin and toxin-coregulated pilus proteins.

This complicated virulence cascade apparently is the result of the acquisition of multiple mobile genetic elements in *V. cholerae*: the *ctx* genes are encoded in a filamentous bacteriophage (18), while the *toxT* and *tcp* genes (including *tcpP*) are located on a large, recently acquired pathogenicity island, which may also be a filamentous bacteriophage (8, 9). The *toxR* gene, however, was apparently present in the ancestral chromosome because it has been found in three other closely related species, *V. fischeri*, *V. parahaemolyticus*, and *Photobacterium profundum* (11, 16, 19). Thus, the recently acquired genetic elements in *V. cholerae* appear to have coerced an ancestral regulatory protein into controlling virulence factor expression. The ancestral role of ToxR was likely as a regulator of outer membrane porins, because *V. cholerae* ToxR still controls expression of the porins OmpU and OmpT in a ToxT- and TcpP-independent manner (1). ToxR of *P. profundum* has also been demonstrated to control outer membrane porin expression (19).

Given the central role ToxR plays in the virulence of *V. cholerae* and the interesting topology of the protein itself, we wished to compare various ToxR sequences to determine (i) if *toxR* is, in fact, an ancestral *Vibrio* gene (i.e., is it widespread throughout *Vibrio* and *Photobacterium* species?), (ii) whether any clues to ToxR function can be drawn from a comparative analysis of multiple ToxR sequences, and (iii) if differences in the *toxR* sequence can be used to distinguish various *Vibrio* and/or *Photobacterium* species.

Identification of *toxR* in seven *Vibrio* and *Photobacterium* species. We designed degenerate oligonucleotides based on conserved regions of the four ToxR protein sequences available. These oligonucleotides, recognizing the coding sequences for EQGFEVDD (located within the transcription activation domain) and VIATGGQN (located in the periplasmic domain), were used to amplify internal *toxR* fragments from an additional seven *Vibrio* or *Photobacterium* species by PCR; the primers also incorporated restriction sites for *Eco*RI and *Bam*HI. The PCR with *Taq* DNA polymerase consisted of 92°C for 45 s, 42°C for 1 min, and 72°C for 1.5 min for 30 cycles. The resulting fragments were first digested with *Eco*RI and *Bam*HI and then ligated into pTZ19U (12) that had been similarly digested.

We amplified partial *toxR* sequences from the human pathogens *V. alginolyticus*, *V. mimicus*, *V. fluvialis*, *V. hollisae*, and *V. vulnificus* and from the fish pathogens *Photobacterium damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida*. Clearly,

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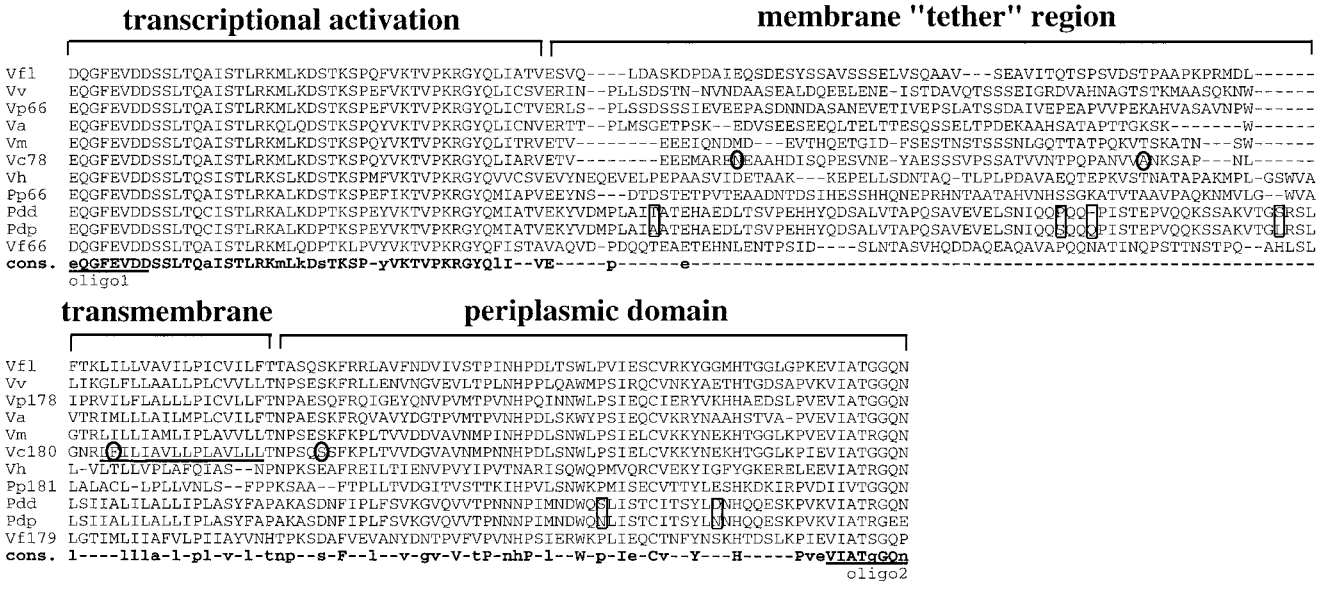

FIG. 1. Alignment of ToxR amino acid sequences from 11 *Vibrio* and *Photobacterium* species. The partial *toxR* genes from *V. fluvialis* (Vfl), *V. vulnificus* (Vv), *V. alginolyticus* (Va), *V. mimicus* (Vm), *V. hollisae* (Vh), *P. damselae* subsp. *damselae* (Pdd), and *P. damselae* subsp. *piscicida* (Pdp) were amplified by using degenerate oligonucleotides recognizing the sequences EQGFEVDD and VIATGGQN (underlined; oligo1 and oligo2). The deduced amino acid sequences are shown aligned with the ToxR amino acid sequences from *V. cholerae* (Vc78 and Vc180; accession no. M21249), *V. parahaemolyticus* (Vp66 and Vp178; accession no. L11929), *V. fischeri* (Vf66 and Vf179; accession no. L29053), and *P. profundum* (Pp66 and Pp181; accession no. U77060); the numerical part of each designation is the starting amino acid residue of the ToxR sequence. The alignment shown was created by MULTALIN, which depicts gaps as dashes (2). The consensus (cons.) sequence is shown at the bottom in boldface letters, residues identical in at least 10 of the 11 sequences are shown in uppercase letters, and residues identical in at least 6 of the 11 sequences are shown in lowercase letters. Residues identical in five or fewer sequences are depicted as dashes. The defined transmembrane domain of *V. cholerae* ToxR is underlined; all other ToxR sequences were predicted to have transmembrane domains in this region by the dense alignment surface method of transmembrane prediction (3). The four amino acids which differ between the classical and El Tor *V. cholerae* biotype ToxR proteins are circled, and the six amino acid differences between *P. damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida* are boxed.

toxR is an ancestral gene of the *Vibrio-Photobacterium* lineage since it is present in all of these species and subspecies.

Alignment of ToxR amino acid sequences reveals a degenerate tether region. The amino acid sequences deduced from these seven partial *toxR* genes were aligned with the previously described ToxR amino acid sequences of *V. cholerae*, *V. fischeri*, *V. parahaemolyticus*, and *P. profundum* (Fig. 1). Interestingly, while there is a high level of homology between the transcriptional activation domains of the ToxR proteins and relatively conserved homology between the transmembrane and periplasmic domains, there is essentially no homology within the region between the transcriptional activation domain and the transmembrane domain (see the consensus sequence [cons.] in Fig. 1). This region tethers the transcriptional activation domain to the cytoplasmic membrane, and we have therefore named it the membrane tether. We utilized several different alignment programs (CLUSTALW, MULTALIN, and BLAST), but none assigned any significant homology within this region between the *Vibrio* and *Photobacterium* ToxR sequences. Also noticeable in the alignment are multiple deletions and/or insertions within this membrane tether region.

The differences between the ToxR proteins is particularly noticeable in closely related bacteria. The closely related subspecies *P. damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida*, which have 100% identical 16S RNA genes, have six amino acid differences in their deduced ToxR sequences, and four of the six differences lie in the membrane tether region (including a 3-bp deletion in *P. damselae* subsp. *damselae* which results in a one-amino-acid deletion [Fig. 1, boxes]). The even more closely related classical and El Tor *V. cholerae* biotype ToxR proteins differ by four amino acids (4), and two of these four differences lie within the membrane tether region (Fig. 1, circles).

The high level of divergence within this membrane tether region between closely related species, subspecies, and biotypes indicates either a strong selective pressure toward divergence of function or an absolute lack of critical function in this region. We favor the latter hypothesis, given the apparent randomness of the amino acid sequences in this region and the insertions and/or deletions occurring here. If this region serves no function besides acting to tether the transcriptional activation domain to the transmembrane domain, then the amino acid sequence would be irrelevant and deletions or insertions would be tolerated. It has been shown that the isolated cytoplasmic portion of *V. cholerae* ToxR is inactive, but fusing the cytoplasmic portion to a heterologous transmembrane domain restores activity (15), consistent with the idea that tethering the cytoplasmic portion to the membrane is critical for ToxR activity. Our prediction that the amino acid sequence of the membrane tether region is irrelevant awaits further structure-function studies.

Use of *toxR* nucleotide divergence for identification of *Vibrio* and *Photobacterium* species. The membrane tether region may serve as a useful diagnostic tool for the distinction of various *Vibrio* and *Photobacterium* species, given the high level of divergence within this region. Using the nucleotide sequences of the partial *toxR* genes, we constructed a phylogenetic tree (Fig. 2A) which demonstrates much greater divergence (based on nucleotide differences) than a phylogenetic tree constructed with the 16S genes from the same species and subspecies (Fig. 2B). For example, *V. cholerae* and *V. mimicus*, which have 99.6% identical 16S genes, have only 71.2% identical nucleo-

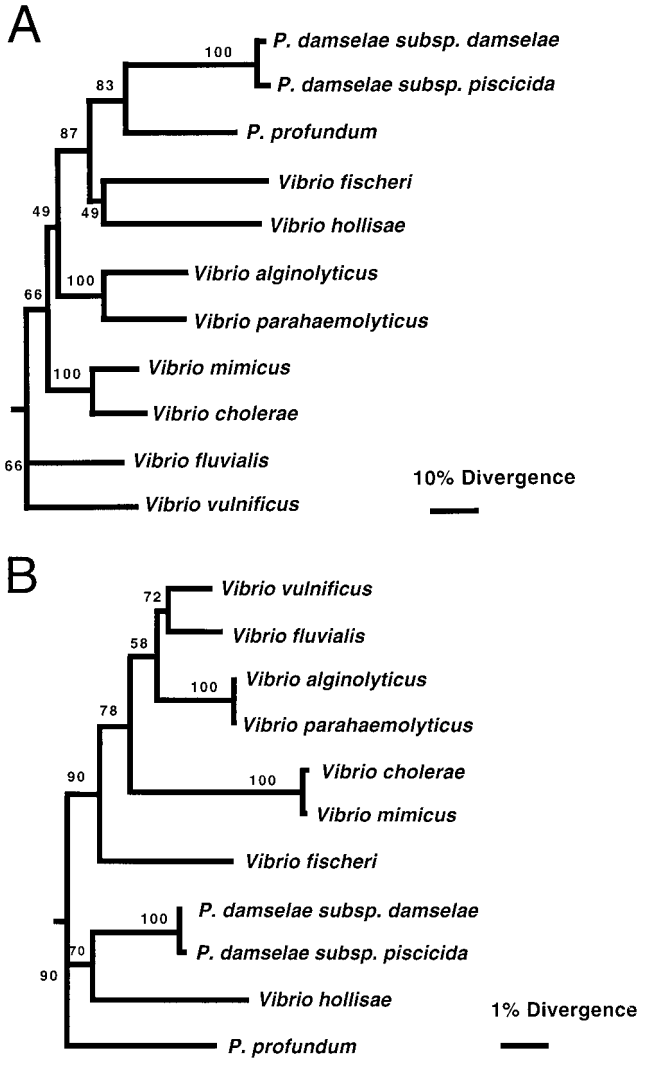

FIG. 2. Unrooted trees constructed by the neighbor-joining method showing the phylogenetic interrelationships of the different *Vibrio* and *Photobacterium* species used in the present study, based on *toxR* gene nucleotide sequences (A) and 16S rRNA genes (B). Bootstrap values (from 1,000 tree replicates generated by using the programs SEQBOOT, DNADIST, and CONSENSE of the PHYLIP package) are given at the branching points. The bars show sequence divergence (6) (note the difference between the scales). Accession numbers for 16S genes: *P. damselae* subsp. *damselae*, X74700; *P. damselae* subsp. *piscicida*, Y18496; *V. alginolyticus*, X74690; *V. fischeri*, X74702; *V. fluvialis*, X76335; *V. hollisae*, X74707; *V. mimicus*, X74713; *V. parahaemolyticus*, X74720; *V. vulnificus*, X76334; *V. cholerae*, X76337; *P. profundum*, D21226. Accession numbers for *toxR* sequences are given in the text and the legend to Fig. 1.

tides within the partial *toxR* sequence described here and *V. alginolyticus* and *V. parahaemolyticus*, which have 99.8% identical 16S genes, have only 61.7% identical nucleotides within the partial *toxR* sequence. This may be a useful means of distinguishing between these species, as suggested by Kim et al. (10). Finally, *P. damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida* have 100% identical 16S genes, as mentioned above (14), but have only 91% identical nucleotides within the partial *toxR* sequence. These *Photobacterium* subspecies have different host specificities, and new molecular techniques to distinguish them would benefit fish disease management strategies. Perhaps divergence within the membrane tether of ToxR could provide the basis of the distinction of other *Vibrio* and *Photobacterium* species.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper were deposited in the GenBank database under accession no. AF170885 (*V. fluvialis*), AF170883 (*V. vulnificus*), AF170882 (*V. alginolyticus*), AF170881 (*V. mimicus*), AF170884 (*V. hollisae*), AF170886 (*P. damselae* subsp. *damselae*), and AF170887 (*P. damselae* subsp. *piscicida*).

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