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Cloning of the Cytotoxin-Hemolysin Gene of *Vibrio vulnificus*

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Genes encoding the cytotoxin-hemolysin of *Vibrio vulnificus* were cloned in *Escherichia coli* by using the lytic cloning vector, λ1059. Subcloning in plasmid pBR325 resulted in the isolation of a 3.2-kilobase DNA fragment containing the cytotoxin gene. By using this fragment as a DNA probe, homologous gene sequences were detected in all 54 *V. vulnificus* strains studied; homologous sequences were present in none of 96 isolates from 29 other bacterial species.

Vibrio vulnificus is a highly virulent estuarine bacterial species that has been implicated as a cause of gastroenteritis, serious wound infections, and a syndrome of primary septicemia with a mortality rate of >50% (1, 11). *V. vulnificus* strains produce an extracellular cytotoxin (cytolysin) that may contribute to the virulence of the organism (3, 9), and purified cytotoxin preparations have been shown to be cytotoxic to Chinese hamster ovary (CHO) cells, hemolytic for mammalian erythrocytes, and lethal for mice (intravenous 50% lethal dose, 80 ng) and to increase vascular permeability in guinea pig skin (3, 9). Whereas most clinical strains have been reported to produce cytotoxin when grown under appropriate in vitro conditions, one study suggested that some environmental isolates possess decreased or absent cytotoxicity (D. L. Tison, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, Q37, p. 210). Production of cytotoxin-hemolysins has been described for a number of other *Vibrio* species (6, 8, 16, 18). The relationship between these cytotoxins and the *V. vulnificus* cytotoxin has not been studied; it is not known whether this cytotoxin is unique to *V. vulnificus*, or even whether all *V. vulnificus* strains produce the same cytotoxin. We cloned the *V. vulnificus* cytotoxin-hemolysin gene in *Escherichia coli* and examined its distribution among isolates from a number of bacterial species.

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DNA from *V. vulnificus* EDL-174 (isolated from a patient with septicemia [15]) was used to construct a genomic library. Chromosomal DNA was purified on a CsCl gradient, partially digested with *Sau*3A (Bethesda Research Laboratories, Inc.), and size fractionated on a sucrose gradient, as described by Hull et al. (5). Fragments in a size range of 8 to 14 kilobases (kb) were ligated into the *Bam*HI sites of λ1059 (7) and packaged in vitro into λ phage (2). Based on reports suggesting that cytotoxin preparations had hemolytic activity (9), the gene library was screened for clones by using a hemolysin plate assay. Phage were preincubated with *E. coli* Q359 (7) and added to NZamine-yeast extract (NZY) top agar (2a) containing 10% (vol/vol) washed rabbit erythrocytes. This mixture was poured onto 0.8% agar plates made with 50 mM Tris hydrochloride (pH 7.4) containing 0.85% NaCl. Plates were incubated overnight at 37°C in a humidified atmosphere of 6% CO_2 in air. Two hemolytic clones, detected by the clearing of erythrocytes around a plaque, were identified out of the first 500 recombinant phage screened.

Recombinant hemolytic phage were absorbed to strain Q359 and incubated until lysis occurred, and the filtered lysates (0.22-μm [pore size] filter; Nalgene Labware Div., Nalge/Sybron Corp.) were examined for cytotoxic and hemolytic activities. Hemolytic activity was measured by a modified microtiter assay (17) by using 96-well U-bottomed microtiter plates. Samples (100 μl) were combined with 100 μl of 50 mM Tris hydrochloride (pH 7.4) containing 0.85% NaCl and 0.25% (vol/vol) washed rabbit erythrocytes. Hemolysis was detected visually by lack of an erythrocyte pellet in a well after overnight incubation at 37°C in 6% CO_2. Cytotoxicity was assayed with CHO cells (4). In tissue culture microtiter plates, samples (20 μl) were combined with 10^3 CHO cells in 200 μl of F12 medium. Cytotoxicity was detected microscopically by the lack of growth or the presence of lysed, crenated, or pyknotic cells after overnight incubation at 37°C in 6% CO_2. Filtered lysates from both clones were hemolytic for rabbit erythrocytes and cytotoxic to CHO cells. Filtered lysates from nonhemolytic clones and supernatants from sonicated *E. coli* Q359 lacked these activities. Both cytotoxic and hemolytic activities were neutralized by antisera raised to crude *V. vulnificus* cytotoxin preparations.

To permit subcloning of the cytotoxin gene, DNA extracted from one hemolytic phage was digested with *Eco*RI (Bethesda Research Laboratories, Inc.) and ligated into plasmid pBR325. *E. coli* HB101 containing recombinant plasmids was sonicated (six 3-s pulses; Heat Systems-Ultrasonics sonicator), and the filtered sonic extracts were screened for cytotoxicity to CHO cells. This resulted in the identification of a recombinant plasmid (designated pCVD701) that encoded the cytotoxin; the plasmid contained 8 kb of *V. vulnificus* DNA inserted into the *Eco*RI site of pBR325 (Fig. 1). Restriction enzyme mapping of pCVD701 revealed internal *Bam*HI and *Hin*dIII sites. pCVD701 was digested with *Hin*dIII and *Eco*RI and religated into pBR325, resulting in the isolation of pCVD702, an 8-kb plasmid with a 3.2-kb *V. vulnificus* DNA insert. Broth cultures of *E. coli* HB101 containing pCVD702 were sonicated, and filtered sonic extracts were found to be cytotoxic for CHO cells and hemolytic for rabbit erythrocytes. Both cytotoxic and hemolytic activities were neutralized by antisera to crude *V. vulnificus* cytotoxin and to purified *V. vulnificus* cytolysin (3) provided by Arnold Kreger.

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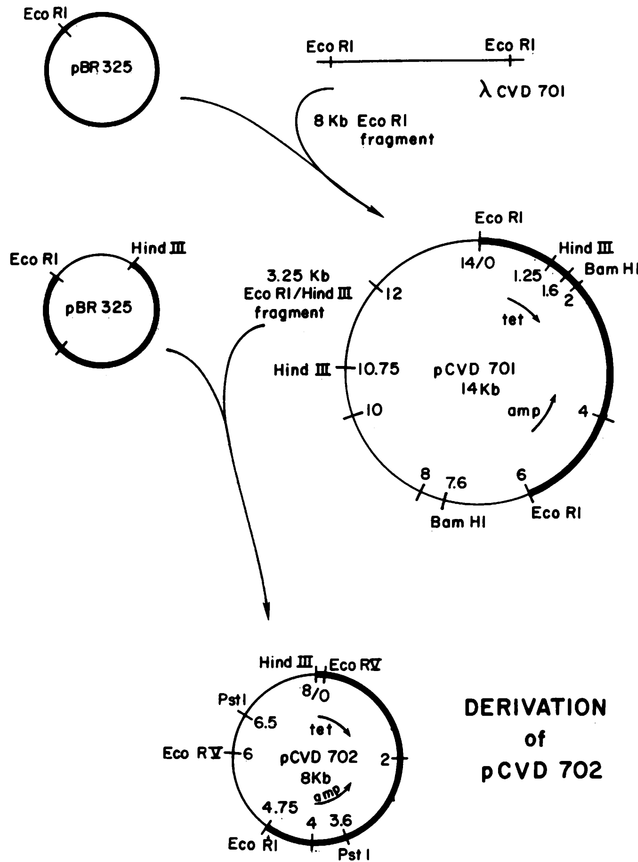

FIG. 1. Derivation of pCVD702.

The 3.2-kb fragment from pCVD702 containing the cytotoxin gene was used to screen 150 isolates from 30 different species for homologous gene sequences. pCVD702 was digested with *Eco*RI and *Hin*dIII, and the 3.2-kb fragment, was isolated and labeled with [α-^{32}P]dATP (New England Nuclear Corp., Boston, Mass.) by nick translation (10). Colony hybridization (13) were performed under high-stringency conditions for *V. vulnificus*, with both high- and low-stringency conditions (12) used for other *Vibrio* species. The results are given in Table 1. All 54 *V. vulnificus* isolates tested (26 environmental and 28 clinical isolates) showed homology with the DNA probe. None of the other 96 isolates, including those from 17 other *Vibrio* and 12 other gram-negative species, had homologous gene sequences. Sixteen randomly selected *V. vulnificus* isolates (eight environmental and eight clinical) were assayed for the production of cytotoxin. Culture filtrates from all strains were cytotoxic for CHO cells and hemolytic for rabbit erythrocytes after growth in heart infusion broth at 37°C, with shaking, for 8 h. The activity of the culture filtrates was in each instance neutralized by antisera to the purified *V. vulnificus* cytolysin.

Based on the ability of specific antisera to neutralize the activity encoded by pCVD702, our cytotoxin appears to be identical to the 56,000-kilodalton *V. vulnificus* cytolysin recently described by Gray and Kreger (3). In screening our gene library, we made use of the observation that purified cytotoxin preparations had hemolytic activity; our work showed that both activities are encoded by a single gene or genes closely linked on the *V. vulnificus* genome. We found that all *V. vulnificus* strains, regardless of source, had gene sequences homologous with the 3.2-kb DNA fragment containing the cytotoxin gene. Although we cannot be certain that the entire gene sequence is intact and expressed by all strains, a random selection of clinical and environmental isolates produced both cytotoxin and hemolysin (neutralizable by specific antisera) under appropriate in vitro conditions.

The production of cytotoxin-hemolysins has been described for a number of *Vibrio* species, including *V. cholerae* (18), *V. damsela* (8), *V. fluvialis* and *V. furnissii* (16), and *V. parahaemolyticus* (6). Although comparative studies are limited, hemolysins in at least two species appear to be related, with *V. hollisae* having gene sequences homologous with the thermostable direct hemolysin of *V. parahaemolyticus* (14). In contrast, the *V. vulnificus* cytotoxin-hemolysin appears to be unique to *V. vulnificus*; even using conditions of low stringency, we were unable to demonstrate the presence of homologous gene sequences in other *Vibrio* species. Similarly, homologous sequences could not be detected in the isolates of members of the families *Enterobacteriaceae*, *Pseudomonadaceae*, and *Acetobacteriaceae* that we studied. Although further subcloning of the gene and the testing of additional strains will be necessary to confirm

TABLE 1. Number of *Vibrio* spp. and other strains showing homology with *V. vulnificus* cytotoxin probe, by species

Species	No. of strains positive for homology with DNA probe/no. tested (n = 150)
V. vulnificus	54/54
V. parahaemolyticus	0/23
V. cholerae O1	0/4
V. cholerae non-O1	0/6
V. hollisae	0/3
V. mimicus	0/3
V. alginolyticus	0/2
V. harveyi	0/2
V. ordalii	0/2
V. aestuarianus	0/1
V. campbellii	0/1
V. damsela	0/1
V. fluvialis	0/1
V. furnissii	0/1
V. metschnikovii	0/1
V. natriegens	0/1
V. pelagius	0/1
V. proteolyticus	0/1
V. splendidus	0/1
Pseudomonas aeruginosa	0/4
Providencia stuartii	0/3
Proteus mirabilis	0/6
Serratia marcescens	0/3
E. coli[a]	0/10
Morganella morganii	0/3
Acinetobacter calcoaceticus	0/1
Klebsiella pneumoniae	0/1
Klebsiella oxytoca	0/1
Enterobacter cloacae	0/1
Citrobacter diversus	0/4
Salmonella spp.	0/4

[a] Includes enterotoxigenic and enteropathogenic strains.

these results, our data suggest that the cytotoxin gene serves as a sensitive and specific marker for *V. vulnificus*.

V. vulnificus EDL-174 was obtained from the Enteric Diseases Laboratory, Centers for Disease Control. Other *V. vulnificus* isolates were obtained from the Centers for Disease Control; Andy Depaola and Gerard Stelma, U.S. Food and Drug Administration; J. D. Oliver, University of North Carolina at Charlotte; and David Tison, University of Texas Medical Branch, Galveston. We thank Patrick Wood for technical assistance with the study.

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