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Medium-Dependent Production of Extracellular Enterotoxins by Non-O-1 *Vibrio cholerae*, *Vibrio mimicus*, and *Vibrio fluvialis*†

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Fluid accumulation at 4 h in the intestines of suckling mice enabled us to distinguish non-O-1 *Vibrio cholerae*, *V. mimicus*, and *V. fluvialis* clinical isolates from environmental isolates. Enterotoxin production was culture medium dependent. Filtrates of cultures grown in tryptic soy broth without glucose but with added 0.5% NaCl did not exhibit marked enterotoxin activity in the assay. Culture filtrates of all clinical strains grown in brain heart infusion broth supplemented with 0.5% NaCl induced large amounts of fluid accumulation in mouse intestines. However, most environmental strains grown in brain heart infusion broth amended as described above were unable to induce fluid accumulation. The enterotoxin present in culture filtrates lost activity at 56°C and appeared to be distinct from previously described virulence factors, including the well-described cholera toxin. The new enterotoxin could represent an important virulence mechanism common to all three species.

Emerging water- and foodborne vibrios other than O-1 *Vibrio cholerae* that cause disease include non-O-1 *V. cholerae* (4, 21), *V. fluvialis* (4, 13), and *V. mimicus* (7), which is biochemically similar to *V. cholerae*. These organisms are usually found in marine or estuarine environments, and their mechanisms for establishing gastroenteritis are not definitely established. A few non-O-1 *V. cholerae* strains produce cholera or cholera-like toxin (6, 16, 22), although most clinical and environmental strains do not (15, 20, 21). Clinical non-O-1 *V. cholerae* isolates have been shown to induce diarrheal disease, even though the genes encoding cholera toxin have not been demonstrable by DNA hybridization (12). Other extracellular products produced by non-O-1 *V. cholerae* have been reported, including permeability and hemorrhagic factors (3, 5, 6, 11, 16, 19, 20) and factors toxic to Y-1 adrenal cells (11, 20) and CHO cells (20). Similarly, *V. fluvialis* also produces factors which induce fluid accumulation in rabbit ileal loops (1, 17, 18, 20), factors which are toxic to Y-1 adrenal cells (18) and CHO cells (14), and hemorrhagic factors (20).

Evidence for an enterotoxin should be based at least on documented in vivo intestinal fluid accumulation in an acceptable animal model. Fluid accumulation in suckling mouse intestines was originally used to detect *Escherichia coli* extracellular heat-stable enterotoxin (ST) (8, 9). This assay was subsequently used to monitor enterotoxin production by non-O-1 *V. cholerae* (15) and *V. fluvialis* (10) clinical isolates. However, there was no evidence of enterotoxin production in this bioassay. Therefore, the search for virulence mechanisms which could account for human diarrhea caused by cholera toxin-negative strains of non-O-1 *V. cholerae* and other *Vibrio* species is important.

Our research indicates that heat-labile extracellular enterotoxins, which are distinct from cholera toxin, were produced almost exclusively by non-O-1 *V. cholerae*, *V. mimicus*, and *V. fluvialis* clinical strains, as demonstrated by the suckling mouse assay. Enterotoxin production was influenced by the culture medium used to grow the test organisms.

MATERIALS AND METHODS

Bacterial cultures. The origins of strains used in this study are listed in Table 1. Cultures previously identified as non-O-1 *V. cholerae* were tested for sucrose fermentation and the Voges-Proskauer reaction, and sucrose-negative and Voges-Proskauer-negative strains were identified as *V. mimicus* (7).

Growth of cultures. Stock cultures were stored at −80°C. Frozen cultures were revived on tryptic soy agar (Difco Laboratories) plus 0.5% NaCl, with incubation at 25°C for 24 h. In preparation for animal virulence assays, cultures were grown at 25°C for 12 h in tryptic soy broth (Difco) without dextrose but with 0.5% NaCl added (broth thus amended is hereafter abbreviated TSB) or brain heart infusion (Difco) broth

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TABLE 1. FA ratios for suckling mice at 4 h after oral administration of sterile culture filtrates of *V. cholerae*, *V. mimicus*, and *V. fluvialis* grown in different media

Species and strain[a]	Origin[b]	Presence of cholera toxin genes[c]	FA ratio obtained with[d]: TSB	FA ratio obtained with[d]: BHI
V. cholerae O-1				
569B	C (India)	+	0.0708	0.0711
SG-N-7277	E (Estuary water, Louisiana)	+	0.0700	0.0711
SG-N-7077	E (Crab, Louisiana)	−	0.0714	0.0687
V. cholerae non-O-1				
N-2030H	C (Louisiana)	−	0.0673	0.1716
N-3	E (Crab, Louisiana)	−	0.0838	0.0759
WA-0-001	E (Estuary water, Oregon)	−	0.0761	0.0690
WA-0-028	E (Estuary water, Oregon)	−	0.0713	0.0680
V. mimicus				
N-2002H	C (Louisiana)	+	0.0689	0.1392
N-2011H	C (Louisiana)	+	0.0761	0.1321
N-2031H	C (Louisiana)	−	0.0893	0.1601
N-53	C (Louisiana)	−	0.0733	0.1367
V-15	E (Chesapeake Bay, Maryland)	−	0.1833	0.1820
V. fluvialis				
DJVP6957	C (Indonesia)	−	0.0595	0.1365
DJVP7225	C (Indonesia)	−	0.0742	0.1232
H-5	E (Estuary water, Maryland)	−	0.0677	0.0731
LSU 9-26a	E (Crab, Lousiana)	−	0.0711	0.0794
LSU 10-41c	E (Crab, Louisiana)	−	0.0880	0.1155
Control (sterile culture medium)			0.0686 ± 0.0061	0.0742 ± 0.0010

[a] The test organisms were grown for 18 h at 37°C and 200 rpm.
[b] C, Clinical isolate (diarrheal stool); E, environmental isolate.
[c] Genes hybridizing to *E. coli* heat-labile enterotoxin gene probe (J. B. Kaper, personal communication).
[d] Each of five 3-day-old mice received 0.1 ml of culture filtrate intragastrically. The FA ratios were determined at 4 h post-inoculation.

with 0.5% NaCl added (hereafter BHI). We transferred 0.1-ml portions of the cultures into 40-ml portions of TSB or BHI in 250-ml Erlenmeyer flasks, which we incubated with shaking (200 rpm) at 37°C until late stationary phase (18 h). Cultures were centrifuged with a clinical centrifuge (Microfuge; Beckman Instruments, Inc.) and filtered through a 0.2-μm sterile membrane filter (Acrodisc; Gelman Sciences, Inc.). Culture filtrate activity was usually tested immediately in suckling mice. However, the activity was stable for at least several months of storage at −20 and −80°C.

Suckling mouse assays. Three-day-old CF-1 mice were obtained from Laboratory Animal Resources, Oregon State University. Mice were submitted to experiments within 2 h after removal from their mothers. Four or five animals were used for each test. We administered a 0.1-ml portion of the culture filtrate containing Evans blue dye (0.01%) into the stomach of each test animal with polyethylene intramedic tubing (PE60; Clay Adams). Inoculated mice were held at 25°C for 4 h and then decapitated. The intestines and stomachs were removed, and the ratio of the pooled intestine-stomach weight to the remaining body weight was measured to calculate the fluid accumulation (FA) ratio, by a modification of the method of Baselski et al. (2).

Heat inactivation. Loosely capped tubes with culture filtrates (1 ml) were heated to 56°C in a water bath or boiled (100°C) for 30 min, and the FA ratios induced by heated and unheated culture filtrates were determined for suckling mice to test the effect of heating on enterotoxin activity.

RESULTS

FA ratios for suckling mice, determined after oral administration of culture filtrates, are summarized in Table 1. The upper limit of the FA ratio confidence interval was 0.0864 ($P = 0.01$) for the sterile TSB medium control and 0.0799 ($P = 0.01$) for the sterile BHI control. FA ratios for mice given culture filtrates of all TSB-grown

TABLE 2. Effect of heat treatment on enterotoxin activity in *Vibrio* and enterotoxigenic *E. coli* culture filtrates

Test organism[a]	FA ratio induced by culture filtrate that was:		
	Unheated	Heated	
		56°C, 30 min	100°C, 30 min
Non-O-1 *V. cholerae* N-2030H	0.1400	0.0719	0.0796
V. mimicus N-53	0.1316	0.0707	0.0721
V. fluvialis DJVP6957	0.1527	0.0701	0.0749
V. mimicus V-15	0.1975	0.1651	0.1426
E. coli H10407 (clinical)[b]	0.1650	0.1869	0.1372
Control (sterile culture medium)	0.0712		

[a] The test organisms were grown in BHI for 18 h at 37°C and 200 rpm.
[b] Produces both ST and heat-labile enterotoxin.

organisms except *V. mimicus* V15 were lower than the upper limit for mice given the sterile TSB control. When grown in BHI, however, all non-O-1 *V. cholerae*, *V. mimicus*, and *V. fluvialis* clinical strains induced very high FA ratios, ranging from 0.1155 to 0.1716. None of the culture filtrates of the *V. cholerae* and *V. fluvialis* environmental isolates except strain LSU 10-41C induced FA ratios above the upper limit of the BHI control (0.0799). The culture filtrate of *V. mimicus* environmental strain V-15, known to produce a toxin similar to the ST of *E. coli* (20), produced an extremely high FA ratio when grown in either TSB or BHI. None of the culture filtrates of O-1-*V. cholerae* strains, regardless of their genetic capability to produce cholera toxin, induced fluid accumulation in suckling mice in 4 h.

The culture filtrate of one clinical strain of each species was heated at 56 and 100°C for 30 min. The enterotoxin in the culture filtrate was inactivated at 56 and 100°C after 30 min (Table 2). The enterotoxins produced by each of these organisms were similar with respect to thermal stability. On the other hand, the ST-like enterotoxin produced by *V. mimicus* V-15 and the ST of *E. coli* H10407 were stable at 56 as well as 100°C.

DISCUSSION

Several media were used for stimulating the production of FA activities in culutre supernatants from clinical vibrio isolates. These media included Casamino Acids plus yeast extract, syncase, TSB, and BHI. BHI was used in previous studies for detecting virulence factors produced by environmental non-O-1 *V. cholerae* (11). When assayed by the suckling mouse procedure in this study, all non-O-1 *V. cholerae* and *V. fluvialis* clinical isolates produced detectable levels of enterotoxins when they had been grown in BHI but not when they had been grown in other media. The original suckling mouse assay of Dean et al. (8) was developed to detect the *E. coli* ST. This assay was subsequently used unsuccessfully by others to detect enterotoxin production by non-O-1 *V. cholerae* (15) and *V. fluvialis* (10) clinical isolates. In the original assay, TSB without added NaCl was used to grow test organisms, and the standard index for a strongly positive FA ratio for suckling mice was 0.09 or higher. In agreement with the previous studies in which the Dean technique was used (10, 15), none of our non-O-1 *V. cholerae* and *V. fluvialis* strains produced FA ratios of $\geq$0.09 after growth in TSB. Therefore, we suspect that failure to detect intestinal FA activities in those studies was related to culture medium.

There is much confusion in the literature as to whether non-O-1 *V. cholerae* and *V. fluvialis* strains are enterotoxigenic (1, 10, 14, 15, 19, 20). This confusion has resulted in part from the synthesis of other virulence factors (permeability factor, cell elongation factor, hemolysins, etc.) and the differences in bioassay systems (tissue cultures, mice, rabbits), route of culture administration (injection, intragastric), and growth media used. In the present study, the choice of growth medium and the 4-h incubation of suckling mice were crucial in revealing the enterotoxin with potent FA activity. Spira et al. (20) reported that some *V. cholerae* strains isolated from the Chesapeake Bay produce a toxin similar to *E. coli* ST. We found that the environmental strain V-15 studied by these workers is actually sucrose-negative *V. mimicus*, and a recent communication also reported ST production by *V. mimicus* strains (7). The non-O-1 *V. cholerae* and *V. fluvialis* heat-labile enterotoxin reported above was different from the V-15 ST-like toxin: the latter is heat stable and produced extracellularly in TSB.

Baselski et al. (2) have shown that orally administered cholera toxin causes significantly increased intestinal FA in suckling mice at 8 h post-inoculation. Similar observations for the CF-1 mice used in these studies have been made

(M. Nishibuchi and R. J. Seidler, unpublished data). Since FA ratios in the present study were measured at 4 h post-inoculation, there was no detectable influence by cholera toxin. Furthermore, culture filtrates of BHI-grown strains of known cholera toxin producers O-1 *V. cholerae* 569B and SG-N-7277 did not elicit a significant increase in FA ratios. FA ratios induced by *V. mimicus* N-2002H and N-2011H, also known to produce cholera toxin (J. B. Kaper, personal communication), did not exceed the FA ratios induced by *V. mimicus* N-2031H, a cholera toxin nonproducer.

Our recent observations indicate that non-O-1 *V. cholerae* N-203OH enterotoxin is a protein (heat sensitivity, precipitated with ammonium sulfate, behavior on Sephadex, stained with Coomassie blue on polyacrylamide) with a molecular weight of about 40,000.

On the basis of the kinetics of action and heat sensitivity, we determined that similar enterotoxins were produced predominantly by clinical isolates of all three *Vibrio* species studied. The enterotoxin could therefore represent an important virulence mechanism common to all three species.

That occasional environmental organisms have the same or a similar ability to produce enterotoxins as clinical isolates is not surprising. This was evident for *V. fluvialis* LSU 10-41C and *V. mimicus* V-15. The production of enterotoxin by occasional environmental isolates may better establish the public health significance of *Vibrio* species which are abundant in marine or estuarine waters and in marine foods consumed by humans.

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