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Production of Enterotoxin by *Yersinia enterocolitica*

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Forty-three strains of *Yersinia enterocolitica* isolated from children with gastroenteritis and 18 laboratory strains were examined for enterotoxin production by using the infant mouse, Y1 adrenal cell, and rabbit ileal loop assay systems. All clinical isolates and seven laboratory strains were found enterotoxigenic in the infant mouse model, but none of the strains produced toxin in the Y1 adrenal cell system. One enterotoxin-producing strain was also tested by the rabbit ileal loop assay, confirming the results of the infant mouse assay. The enterotoxin was heat-stable and as active in the 6-h rabbit ileal loop as in the 18-h assay, suggesting a similarity to the heat-stable enterotoxin of *Escherichia coli*.

Yersinia enterocolitica infections in humans have been recognized with increasing frequency in recent years. Gastroenteritis is the most common clinical association, particularly in young children (6, 10). Pseudoappendicitis (9), nonsuppurative arthritis (21), septicemia (12, 18), and suppurative infections (15) are also described. At the Montreal Children's Hospital, 92 infants and children during 1974 to 1977 were diagnosed as having *Y. enterocolitica* infection, all but one of whom had gastroenteritis (C. H. Pai, unpublished data). By this estimate, and discounting enteropathogenic serotypes of *Escherichia coli*, *Y. enterocolitica* is second only to *Salmonella* as a cause of sporadic bacterial gastroenteritis of children in our area.

The pathogenic mechanisms of this organism are not known. Carter (3) reported an experimental infection of *Y. enterocolitica* in mice, which resembled a naturally acquired human infection. Early changes in the small intestine included neutrophil infiltration in the Peyer's patches of the distal ileum followed by local abscess. Extraintestinal dissemination resulted in abscess formation in the liver, spleen, and lungs. However, a detailed review of 23 cases of childhood *Y. enterocolitica* enteritis seen at this hospital has shown that the clinical symptoms of *Y. enterocolitica* gastroenteritis are much milder than would be expected from the pathological findings in the mouse model (O. Hammerberg and C. H. Pai, unpublished data). Furthermore, a significant number of our patients had profuse watery diarrhea as a major symptom, suggesting a similarity between gastroenteritis due to *Y. enterocolitica* and that caused by enterotoxigenic strains of *E. coli*. This led us to investigate the possibility of enterotoxin production as a pathogenic mechanism in human gastroenteritis due to *Y. enterocolitica*. In this communication, we report the presence of enterotoxin activity in culture filtrates of *Y. enterocolitica*.

MATERIALS AND METHODS

Bacterial strains. Sixty-one strains of *Y. enterocolitica* were examined: 43 strains (Montreal Children's Hospital [MCH] strains) were isolated from children with gastroenteritis seen at the Montreal Children's Hospital during a 3-year period (from July 1974 to June 1977), and 18 strains (National Reference Centre [NRC] strains) were obtained from S. Toma, the National Reference Centre for *Yersinia*, Laboratory Services Branch, Ontario Ministry of Health, Toronto, Ontario, Canada. The MCH strains were all cultured from stool specimens. Identification was carried out by using the differential characteristics defined by Sonnenwirth (17), and sero- and biotyping were performed by the Provincial Laboratory, Ste. Anne de Bellevue, Quebec, Canada. All of these isolates were of serotype O:3, biotype 4. The NRC strains were isolated originally from nonhuman sources, except for 3 strains (2 from stools, 1 from urine), and included 10 nontypable strains and one each of serotypes O:1, O:5, O:6,31, O:8, O:10, O:11,23, O:16, and O:13,7. Of the 18 strains, 16 were of biotype 1 and the other two were of biotypes 2 and 3. Among the 10 nontypable strains, 5 were rhamnose positive and, hence, biochemically atypical (1, 2). The MCH strains were kept at −20°C in glycerol, and the NRC strains were stored as stab cultures.

Preparation of culture filtrates. Organisms were inoculated into 25 ml of media containing 2% Casamino Acids (Difco), 1% yeast extract (Difco), and 0.4% glucose (pH 8.5) in 250-ml Erlenmeyer flasks. Unless otherwise stated, all *Y. enterocolitica* cultures were incubated on a rotary shaker (200 rpm) at room temperature (22 to 26°C) for 48 h and then centrifuged at 12,000 × *g* for 10 min. The supernatant fluids were filtered through Gelman filters (pore size, 0.45 μm) and assayed immediately for enterotoxigenic activity or stored at 4°C for toxin assay within 3 days or at −20°C

for longer storage (up to 10 days). Under these conditions, no loss of enterotoxigenic activity was observed. Culture filtrates of *E. coli* strains were prepared in the same way, except that incubation was at 37°C for 24 h.

Enterotoxin assay. Enterotoxin activity of culture filtrates was assayed by using infant mouse (5, 13), rabbit ileal loop (7), and Y1 adrenal cell (14) systems. Y1 adrenal tumor cells were obtained from D. Sack and maintained in Ham F10 medium supplemented with horse serum (15%), and fetal calf serum (2.5%). The tissue culture was subcultured into 96-well miniculture plates, and assays were performed when a monolayer was formed, usually in 2 to 3 days. Samples were tested in duplicate, and the cells were observed in 4 to 6 h for typical rounding. Three-day-old suckling mice (Charles River CD-1, outbred albino) and rabbits (albino, New Zealand) weighing 3 to 4 pounds (ca. 1.36 to 1.81 kg) were obtained from Canadian Breeding Farm Laboratories, Ltd., St. Constant, Quebéc. For the infant mouse assay, 0.1 ml of culture filtrate was injected through the abdominal wall into the milk-filled stomach of each of three mice. Four hours later, the mice were killed and the entire intestine was removed. The intestine and remaining body were weighed to calculate the ratio of (intestine weight)/(remaining body weight). The rabbit ileal loop assay was performed as described by Evans et al. (7). A 1-ml sample of sterile culture filtrate was injected into 5-cm ileal loops in duplicate in each animal, and the volume/length ratio of each loop was determined at 6 or 18 h postinjection. For each assay culture, filtrates of the following strains of *E. coli* were included as positive and negative controls: TD 218-C1 (heat-labile enterotoxin), TD 213-C5 (heat-stable enterotoxin), and 185 (nontoxigenic). These strains were obtained from David Sack of the Johns Hopkins University School of Medicine, Baltimore, Md.

Enterotoxin titer. Relative activities of enterotoxin were measured in the infant mouse assay system by diluting culture filtrates in isotonic buffered saline (hemagglutination buffer, Difco). The enterotoxin titer was expressed as the reciprocal of the highest dilution that gave a positive reaction ([intestine weight]/[remaining body weight] ratio of ≥0.083 in the mouse assay).

Invasiveness assay. The ability of *Y. enterocolitica* to invade guinea pig eyes was tested by using the method described by Serény (16). A drop of the suspension containing 5×10^9 cells per ml was inoculated into guinea pig conjunctivae, and the animals were observed daily for 7 days for evidence of keratoconjunctivitis. Two guinea pigs were tested for each specimen. *E. coli* 4608-58 and HS were also tested in each experiment as positive and negative controls, respectively. The strains were obtained from H. L. DuPont of the University of Texas Medical School, Houston, Tex.

RESULTS

Enterotoxigenicity. All of the 43 MCH strains and 7 of the 18 NRC strains were enterotoxigenic in the infant mouse model. This assay distinguished two populations of organisms, clearly separating the positive and negative groups (Fig. 1). Fifty positive cultures had a ratio of (intestinal weight)/(remaining body weight) of ≥0.10 (0.142 ± 0.018), and 11 negative cultures had a ratio of ≤0.07 (0.062 ± 0.002). The positive values were well above 0.083, that accepted as a minimum positive value for *E. coli* heat-stable enterotoxin (13). No enterotoxigenic activity was demonstrable in Y1 adrenal cell assay. Strain MCH 9033, which was positive in the infant mouse assay, was also positive in the 6- and 18-h rabbit ileal loop assay (Table 1). The enterotoxin activity was heat stable. The results suggested that the enterotoxin of *Y. enterocolitica* might be similar to the heat-stable toxin of *E. coli* (7, 11).

Effect of growth temperature on enterotoxin production. When cultures were incubated at 37°C, none of the strains produced enterotoxin. The effect of growth temperature on enterotoxin production was further investigated by growing *Y. enterocolitica* MCH 9033 at various temperatures (Table 2). Enterotoxin activity was demonstrable only when organisms were grown at 30°C or below, with the highest activity at 26°C. Since the amount of growth obtained under these conditions was also inversely related to the growth temperatures, the concentration of enterotoxin in culture filtrates seemed related to the amount of cell growth rather than to growth temperature per se. A number of complex media was used to grow the organisms at 32°C or higher. At these temperatures, none of the media used allowed the organisms to grow to an optical density of 3.5 or greater, even after a prolonged incubation period. All of the culture filtrates were again neg-

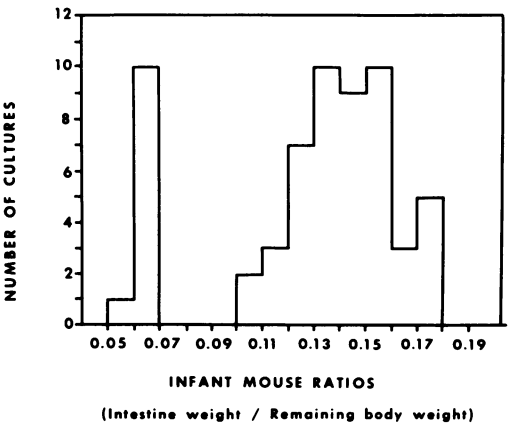

FIG. 1. *Infant mouse test for detection of enterotoxin. Values expressed in the ratios of (intestine weight)/(remaining body weight) were the averages of two to three assays, using three mice per specimen in each assay. Values for control strains of E. coli were 0.149 for TD 213-C5 (heat-stable enterotoxin) and 0.059 for 185 (non-enterotoxigenic).*

TABLE 1. *Results of rabbit ileal loop assays*

Strain	Characteristics[a]	Ratio of volume/length (ml/cm)[b]			
		6 h[c]		18 h[c]	
		Unheated	Heated[d]	Unheated	Heated[d]
Y. enterocolitica					
MCH 9033	MCH isolate	0.42 ± 0.17	0.41 ± 0.09	0.80 ± 0.28	0.61 ± 0.20
E. coli					
TD 218-C1	LT^+	0.16 ± 0.07	0.02 ± 0.015	1.57 ± 0.41	0.19 ± 0.06
TD 213-C5	ST^+	0.41 ± 0.18	0.42 ± 0.08	0.71 ± 0.16	0.89 ± 0.28
185	ENT^-	0.02 ± 0.014	NT[e]	0.02 ± 0.016	NT

[a] LT^+, Heat-labile enterotoxin; ST^+, heat-stable enterotoxin; ENT^-, enterotoxin negative.
[b] Mean values ± one standard deviation. Data were from duplicate loop assays in each of four animals.
[c] Postinjection.
[d] Culture filtrates were boiled for 15 min.
[e] NT, Not tested.

TABLE 2. *Effect of growth temperature on enterotoxin production*[a]

Temp (°C)	Incubation period (h)	OD[b]	Enterotoxin titer[c]
26	24	5.9	8
	48	7.5	128
28	24	5.8	2
	48	7.0	64
30	24	3.7	0[d]
	48	5.5	16
32	24	3.2	0
	48	3.1	0
34	24	2.7	0
	48	2.6	0
37	24	1.9	0
	48	2.1	0

[a] Enterotoxin activity was assayed in infant mice.
[b] Optical densities (OD) of cultures as measured in a spectrophotometer (Spectronic 20, Bausch & Lomb) at 600 nm.
[c] Reciprocal of the highest dilution that gave a positive result in infant mice assay.
[d] No enterotoxin activity in undiluted culture filtrates.

ative for enterotoxin.

Serotypes. No conclusion could be made regarding the relationship of enterotoxicity and serotypes, since only a few strains of *Y. enterocolitica* that belonged to those other than serotype O:3 were tested in this study. However, although all of the 43 MCH strains were enterotoxigenic and belonged to O:3, only 2 of the 10 nontypable strains tested were found to be positive for enterotoxin. Five rhamnose-positive strains that were included among the nontypable strains were all negative for enterotoxin.

Invasiveness. Ten strains from the MCH isolates were tested for invasiveness by the Serény test. All were negative. Variation in the growth conditions (37°C or room temperature; 24 or 48 h of incubation) of inocula did not alter the results.

DISCUSSION

The evidence presented in this paper indicates that the majority of *Y. enterocolitica* was capable of producing enterotoxin. The characteristics of this enterotoxin were similar to those of heat-stable enterotoxin of *E. coli* (7, 11): (i) it was active in the infant mouse and rabbit ileal loop systems, but not in the Y1 adrenal cell assay; (ii) it was stable at 100°C for 15 min; and (iii) it was as active in the 6-h rabbit ileal loop assay as in the 18-h assay. However, in contrast to the finding that only 2 of more than 1,200 isolates of *E. coli* from children with gastroenteritis seen at the Montreal Children's Hospital were enterotoxigenic (C. H. Pai and V. Mors, unpublished data), all of the clinical isolates of *Y. enterocolitica* tested in this study were enterotoxigenic. It is tempting to speculate that enterotoxin production in *Y. enterocolitica* may be controlled by chromosomal gene(s) as in *Vibrio cholerae* (8). Further studies on genetic and biochemical characteristics of enterotoxin production in *Y. enterocolitica* are in progress.

It was of interest that, although all of the clinical isolates (all were of serotype O:3) were enterotoxigenic, only 2 of the 10 nontypable strains tested in this study were found toxigenic. Furthermore, five biochemically atypical strains (rhamnose positive) of this nontypable group were also negative for enterotoxin. In the provinces of Ontario and Quebéc, the large majority of *Y. enterocolitica* isolated from human sources belong to serotype O:3 (19), and nontypable strains are recovered frequently from environmental sources. For example, a monthly report 1977) from the National Reference Laboratory ratory for *Yersinia*, Toronto, Ontario, showed that of 21 *Y. enterocolitica* strains isolated from human sources in Canada, 18 belonged to serotype O:3 and only 2 were nontypable, whereas 28 of 48 strains isolated from nonhuman sources (raw milk, 28; water, 19; and uncooked ham, 1)

were nontypable and none belonged to serotype O:3. In view of the uncertainty regarding the taxonomic status of these biochemically and serologically atypical strains of *Y. enterocolitica* (2, 3), studies on the prevalence and frequency of enterotoxin production among atypical strains may be justified.

Enterotoxin production by *Y. enterocolitica* was demonstrable only when the organisms were grown at 30°C or below. The comparison of cell concentrations and the amount of toxin produced in cultures incubated at various temperatures (Table 2) suggests that the growth of these organisms was favored at the lower temperatures, resulting in the accumulation of enterotoxins in culture supernatant fluids to a level detectable by the available assay techniques. On the other hand, Carter and Collins (4) reported that the growth of a *Y. enterocolitica* strain in intravenously infected mice depended upon the temperature at which the challenge inoculum had been grown in vitro. Organisms cultured at 25°C were cleared from the blood at a slower rate and were more resistant to intracellular killing, as compared to those grown at 37°C. Temperature-dependent characteristics of *Yersinia* include fermentative capability, motility, and growth potential on various media (17). In consideration of the pathogenesis of diarrheal diseases due to *Y. enterocolitica*, one would have to ask whether the enterotoxins produced by these organisms in vitro were also produced in vivo, where temperature would be close to 37°C. An animal model is required to study the role of enterotoxin and the mechanism by which *Y. enterocolitica* causes diarrhea. Enterotoxin production in vivo by *E. coli* has been demonstrated in experimentally infected animals (20).

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ADDENDUM

While this manuscript was being prepared, two reports were presented at the Third International Symposium on *Yersinia*, Mont-Gabriel, Quebéc, Canada, and Saranac Lake, N.Y., 25 to 28 September, 1977, describing enterotoxin production by *Y. enterocolitica* (R. M. Robins-Browne, C. van Vooren, and J. Koorhof, The pathogenesis of *Yersinia enterocolitica* gastroenteritis, and J. C. Feeley, J. Wells, T. Tsai, and N. Puhr, Enterotoxin detection in *Yersinia enterocolitica*).

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