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Production of Heat-Stable, Methanol-Soluble Enterotoxin by *Yersinia enterocolitica*

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Seven isolates of *Yersinia enterocolitica* serotype O:8, recovered during an outbreak of gastrointestinal illness, were examined for enterotoxin production. All seven strains were enterotoxigenic in the suckling mouse model, and three of five isolates tested produced keratoconjunctivitis in the guinea pig eye model (Sereny test). Enterotoxin was detected in broth supernatant fluid after 12 h of incubation at 25°C. The toxin was not inactivated by exposure to 121°C for 30 min or by storage at 4 or −40°C for at least 5 months. The enterotoxin was also acid stable and methanol soluble. Methanol extraction did not affect its heat stability or enterotoxic activity in suckling mice. These physical characteristics plus limited ultrafiltration studies suggest that the enterotoxin is a low-molecular-weight substance. *Y. enterocolitica* enterotoxin resembles *Escherichia coli* heat-stable enterotoxin in heat and pH stability, methanol solubility, and enterotoxic activity in suckling mice. However, its role in the pathogenesis of human diarrhea has not been established.

In 1939 Schleifstein and Coleman (15) described the first recognized cases of gastrointestinal illness due to *Yersinia enterocolitica*. After this initial report, many years elapsed before *Y. enterocolitica* was recognized as an important cause of human diarrheal illness (10). Only recently have isolates from patients with diarrhea been evaluated in standard animal models used to study the invasiveness and enterotoxigenicity of enteric pathogens (14, 17, 18). Feeley et al. (J. C. Feeley, J. G. Wells, T. F. Tsai, and N. D. Puhr, Contrib. Microbiol. Immunol., in press) reported that *Y. enterocolitica* serotype O:8 strains recovered during an outbreak of gastrointestinal illness (2) produced a heat-stable enterotoxin. The toxin was detected in filtrates of broth cultures incubated at 25°C, but not 37°C, for 48 h. All strains were negative for heat-labile enterotoxin in the Y-1 adrenal cell assay. Several of these strains were also capable of penetrating guinea pig conjunctival epithelium (Sereny test). More recently, Pai and Mors (13) found that 24-h broth cultures of *Y. enterocolitica* serotype O:3 isolates contained enterotoxic activity.

This report describes additional studies of (i) the physical characteristics of the *Y. enterocolitica* heat-stable toxin; (ii) factors influencing its production in vitro; and (iii) similarities between *Y. enterocolitica* enterotoxin and the heat-stable enterotoxin (ST) of *Escherichia coli*. (This report was presented in part at the Interscience Conference on Antimicrobial Agents and Chemotherapy, Atlanta, Ga., October, 1978.)

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MATERIALS AND METHODS

Bacterial strains, storage, and culture conditions. Seven strains of *Y. enterocolitica* serotype O:8, biotype 2,1, isolated during an outbreak of food-borne yersiniosis (2), were obtained from James C. Feeley (Center for Disease Control, Atlanta, Ga.). All strains except A2635 (recovered from chocolate milk) were isolated from patients with gastrointestinal illness. *Y. enterocolitica* 7031, a non-enterotoxigenic serotype O:3 strain, was provided by R. M. Robins-Brown (South African Institute for Medical Research, Johannesburg, South Africa). For long-term storage, *Yersinia* isolates were frozen in brain heart infusion broth (BHIB) (BBL Microbiology Systems) plus 15% glycerol and maintained at −40°C, or stored on brain heart infusion agar (BHIA) (BBL Microbiology Systems) slants at room temperature. *E. coli* 431, an ST-producing strain obtained from Harley Moon (National Animal Diseases Center, Ames, Iowa), was stored in Casamino Acids-yeast extract medium (6) plus 15% glycerol at −65°C.

Unless otherwise stated, *Y. enterocolitica* isolates were grown in 10 ml of tryptic soy broth (TSB) (Difco) plus 0.6% yeast extract (Difco) at 25°C with aeration using a shaker water bath at 200 rpm for 24 h. In studies of toxin production under more alkaline conditions, two modifications (TSB-PO_4 and TSB-Tris) of the above TSB medium were used. TSB-PO_4 contained 0.028 M K_2HPO_4 and was adjusted to pH 8.5

with 5 N NaOH before autoclaving. TSB-Tris contained 0.05 M tris(hydroxymethyl)aminomethane-chloride buffer, pH 8.5 (Sigma). In these studies flasks were removed from the incubator at 0, 4, 8, 12, 14, and 16 h, and the optical density, glucose concentration, pH, and enterotoxin activity were determined. Using 0.1 M phosphate-buffered saline (pH 7.2) as a diluent, 1:4 and 1:8 dilutions of the 12-, 14-, and 16-h supernatant fluids were prepared and tested for enterotoxin activity.

Preparation of crude toxin and enterotoxin assay. TSB cultures were centrifuged at 27,700 × *g* for 15 min at 4°C. The supernatant fluid (crude toxin) was removed and stored at 4°C until assayed for enterotoxin activity by using the suckling mouse model of Dean et al. (4). Crude toxin (0.1 ml) was injected percutaneously into the milk-filled stomach of 3- to 5-day-old mice (Timco Breeding Laboratories, Inc., Houston, Tex.). During our early experiments, inoculated mice were held at 37°C for 4 h; however, in later studies, injected mice were held at room temperature for 4 h (11). The animals were sacrificed, and the entire intestine was removed. The intestinal weight and remaining body weight were determined and the gut weight/remaining body weight (GW/BW) ratio was calculated. Ratios of ≥0.083 were considered positive.

Temperature and pH stability of crude toxin. To assess the stability of crude toxin under various storage conditions, samples of heated crude toxin were stored at 4 and −40°C for varying periods of time and then assayed for enterotoxic activity. Also, crude toxin from strains A2627 and A2635 was incubated at 4, 65, 100, and 121°C (autoclaved) for 30 min before testing for enterotoxic activity.

Crude toxin (pH 8.3) from a 24-h broth culture of strain A2627 was tested for pH stability. Samples of crude toxin were membrane filtered (0.22-μm pore size, Millipore Corp.), adjusted to the desired pH with 1 N HCl or 1 N NaOH, incubated for 4 h at 37°C, and then readjusted to pH 8.3 using 1 N HCl or 1 N NaOH. Final volumes were so similar that no volume adjustments were performed. Controls consisted of (i) crude toxin incubated at 37°C for 4 h without pH adjustment; (ii) crude toxin incubated at 100°C for 30 min without pH adjustment; and (iii) uninoculated TSB (incubated at 25°C with aeration for 24 h) adjusted to pH 1.0 with HCl, held at 37°C for 4 h, and readjusted to pH 8.3 with NaOH.

Ultrafiltration studies. Broth cultures of *Y. enterocolitica* 7031 and A2627 were incubated at 25°C with aeration for 24 h. Casamino Acids-yeast extract medium was inoculated with *E. coli* 431 and incubated at 37°C with aeration for 18 h. Supernatants of the *Yersinia* and *E. coli* cultures were prepared as described above and concentrated fivefold with PM-10 or UM-10 ultrafiltration membranes (Amicon Corp.). Retentates and ultrafiltrates were membrane filtered (0.22 μm) and stored at 4°C until tested for enterotoxic activity.

Methanol extraction. Five milliliters of concentrated A2627 crude toxin (UM-10 retentate) was added dropwise to a 15-fold excess of reagent-grade methanol (Fisher) with vigorous stirring and held at 22°C for 1 h. The preparation was centrifuged at 27,150 × *g* for 30 min at 4°C, and the supernatant was removed. The precipitate was washed with an additional 10 ml of methanol and centrifuged as above, and the methanol fraction was added to the supernatant from the first centrifugation. The methanol-soluble fraction was evaporated almost to dryness at 37°C under vacuum and adjusted to a final volume of 5 ml with distilled water. The methanol-insoluble fraction was air dried at room temperature and adjusted to a final volume of 5 ml with distilled water. Both fractions were membrane filtered (0.22 μm) and stored at 4°C. As a control, the above procedures were performed on strain 7031 UM-10 retentate.

Invasiveness assay. The ability of five isolates of *Y. enterocolitica* to invade epithelial cells was tested in the guinea pig eye model (Sereny test) (16). Growth from a 48-h BHIA culture incubated at room temperature was suspended in phosphate-buffered saline. One drop of a suspension containing 1.5×10^{10} to 2.3×10^{10} organisms per ml was inoculated into guinea pig conjunctivae, and the animals were examined daily for 5 days for evidence of keratoconjunctivitis. Each specimen was inoculated into the right eye of two guinea pigs. A known invasive strain, *E. coli* 10673-70, was used as a positive control.

RESULTS

Invasiveness assay. Three of the five *Y. enterocolitica* isolates tested for invasiveness gave positive Sereny tests (Table 1). Conjunctivitis or keratoconjunctivitis developed after 48 to 72 h in the affected guinea pigs. The conjunctivitis caused by the *Yersinia* isolates was usually less severe than that caused by the invasive *E. coli* isolate.

Enterotoxin production. Supernatants of broth cultures of all seven serotype O:8 strains incubated at 25°C with shaking for 48 h con-

TABLE 1. *Enterotoxin production and invasiveness of Yersinia enterocolitica serotype O:8 from humans with gastrointestinal illness*

Isolate	GW/BW ratio[a]	Sereny test[b]
A2611	0.097	Negative
A2621	0.100	ND[c]
A2624	0.117	ND
A2627	0.107	Positive
A2628	0.087	Positive
A2630	0.098	ND
A2635[d]	0.095	Postive
Toxigenic (ST$^+$) *E. coli*	0.114	ND
Nontoxigenic *E. coli*	0.065	Negative
Invasive *E. coli*	ND	Positive

[a] GW/BW ratio using the suckling mouse model assay for enterotoxin. Five mice were used for each test. Ratios of 0.083 or greater are positive.

[b] Guinea pig eye test for invasiveness.

[c] ND, Not done.

[d] Chocolate milk isolate associated with gastrointestinal illness.

tained enterotoxin detectable in the suckling mouse model (Table 1). Incubation of broth cultures for 72 or 96 h did not yield higher GW/BW ratios. Subsequent studies with strains A2611, A2627, A2628, and A2635 revealed that toxin could be detected in supernatant fluid of 24-h TSB cultures. Additional experiments were conducted to determine (i) the onset of toxin production (or release) and (ii) the effect of alkaline conditions on toxin production with buffer- and pH-adjusted TSB. These experiments were performed on two occasions and the results were averaged (Fig. 1). The pH of the TSB cultures reached a value of approximately 6.2 after 9 to 10 h of incubation, but returned to alkaline by 12 h of incubation. TSB-PO_4 and TSB-Tris broth cultures never reached acid conditions during the 24-h period. Most of the glucose in the media was utilized during the first 8 h of incubation (Fig. 1). The 12-, 14-, 16-, and 24-h supernatants from all three media contained enterotoxic activity. At these times the GW/BW ratios of 1:4 dilutions and also of undiluted supernatants of TSB-PO_4 and TSB-Tris were slightly but not significantly higher than the respective TSB supernatants.

Storage in BHIB with 15% glycerol at −40°C for 5 months, and monthly subculture on BHIA slants held at room temperature, did not affect enterotoxin production of four isolates tested (Table 2). However, two of these four isolates apparently lost the ability to produce enterotoxin after storage on BHIA slants at room temperature for 3 months without subculture.

Temperature and pH stability of crude toxin. Exposure of crude toxin from two isolates to 4, 60, 100, and 121°C for 30 min did not appreciably affect its activity in the suckling mouse assay. Long-term storage of crude toxin samples at 4°C resulted in a slight decline in the GW/BW ratios, but ratios were still ≥0.083 after 7 months at 4°C (Table 3). Storage of culture supernatants at −40°C for 5 months did not inactivate the crude toxin. Exposure of crude toxin to pH 1 through pH 11 at 37°C did not affect its enterotoxic activity in suckling mice (Table 4).

Ultrafiltration and methanol extraction.

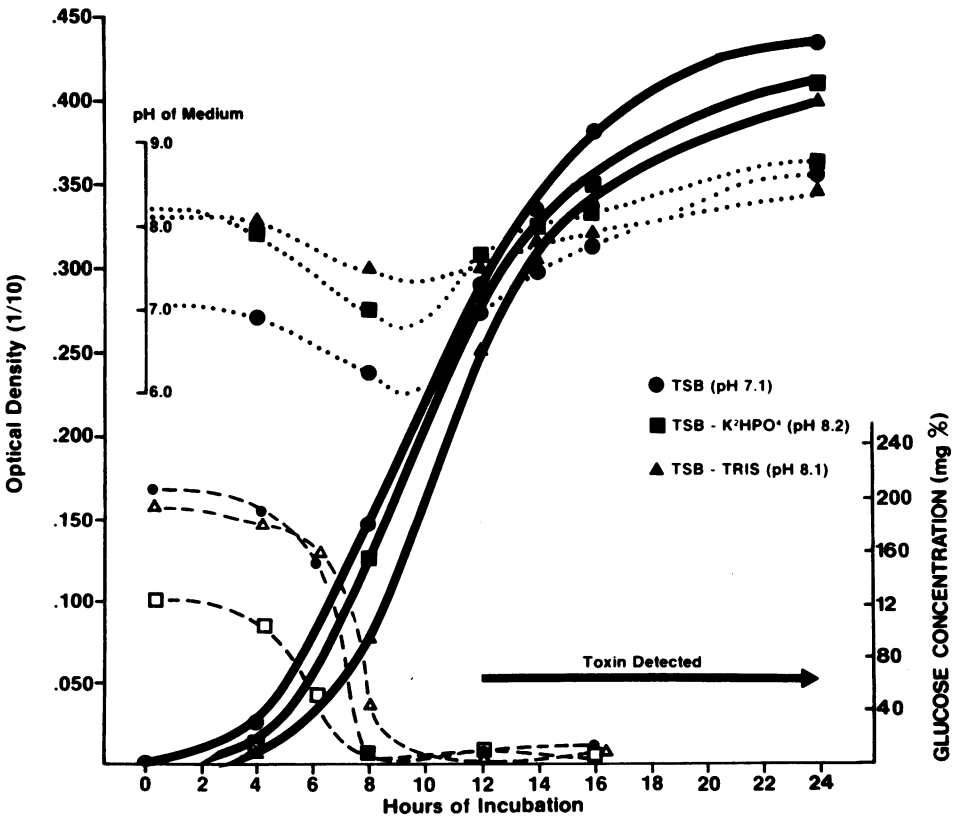

FIG. 1. *Effect of pH on enterotoxin production by Y. enterocolitica. Growth conditions were as described in the text.* (——) *Optical density;* (·····) *pH of medium;* (-----) *glucose concentration of medium.*

When strain A2627 crude toxin was filtered using a PM-10 membrane, all toxin activity remained in the retentate (Table 5). The GW/BW ratio was appreciably higher in the retenate (0.124) than in unfiltered A2627 crude toxin (0.094). *E. coli* ST passed through the PM-10 membrane and was present in both the retentate and ultrafiltrate. Neither the A2627 crude toxin nor *E. coli* ST passed through the UM-10 membrane.

After extraction of the concentrated A2627 toxin (UM-10 retenate) with methanol, enterotoxic activity was present only in the methanol-soluble fraction (Table 6). The methanol-soluble toxin was also heat stable.

TABLE 2. *Stability of enterotoxin production by Y. enterocolitica*

Isolate	GW/BW ratio[a] after:		
	Monthly subculture on BHIA (5 mo)	Subculture on BHIA every 3 mo	BHIB/glycerol storage at −40°C (5 mo)
A2611	0.097	0.090	0.143
A2627	0.107	0.078	0.115
A2628	0.087	0.068	0.127
A2635	0.095	0.095	0.108

[a] GW/BW ratio by the suckling mouse model assay for enterotoxin. Four to five mice were used per test. Ratios of 0.083 or greater are positive.

TABLE 3. *Effect of various storage conditions on Y. enterocolitica crude toxin*

Isolate	Fresh supernatant (crude toxin) (GW/BW ratio)	GW/BW ratio[a] after storage:			
		4°C			−40°C
		1 mo	4 mo	7 mo	5 mo
A2627	0.117	0.117	0.112	0.108	0.108
A2628	0.126	ND[b]	0.114	0.094	ND
A2635	0.115	0.112	0.107	0.103	0.110

[a] GW/BW ratio, determined by the suckling mouse model assay for enterotoxin. Four to six mice were used per test. Ratios of ≥0.083 are positive.

[b] ND, Not done.

DISCUSSION

These studies confirm the observation of Feeley et al. (Contrib. Microbiol. Immunol., in press) that *Y. enterocolitica* serotype O:8, biotype 2,1, from humans is invasive and produces a heat-stable enterotoxin. Similarly, Pai and Mors (13) have reported that serotype O:3, biotype 4, isolates recovered from Canadian children with gastroenteritis produced a heat-stable enterotoxin. Unlike Pai and Mors, who reported enterotoxic activity in 48- and 24-h culture supernatants, respectively, we detected toxin in the supernatant of 12-h broth cultures. Our ability to detect toxin earlier may be related to differences in the agitation systems used.

The factors that promote early production or release of *Yersinia* enterotoxin in vitro are poorly understood. Studies of *E. coli* ST production in complex media suggest that medium pH and glucose content may control synthesis or release of enterotoxin. Johnson and Johnson (7) found that *E. coli* ST could be detected during the early logarithmic phase of growth and that pH control (pH 8.5) resulted in greater levels of ST at all stages of growth. In our studies of *Y. enterocolitica*, enterotoxin was not detected in culture supernatants until the late logarithmic phase of growth. Although TSB-PO_4 and TSB-Tris media (which remained alkaline throughout the 24-h incubation period) yielded slightly higher GW/BW ratios, they did not appear to promote early synthesis or release of enterotoxin. Perhaps strict pH control may have yielded different results.

Several authors (1, 7) have noted that glucose appears to have an inhibitory effect on *E. coli* ST production, especially when cultures are grown in defined media. Similarly, enterotoxic activity did not appear in *Y. enterocolitica* cul-

TABLE 4. *Effect of pH on Y. enterocolitica crude toxin activity*[a]

pH	GW/BW ratio[b]	No. of mice	pH	GW/BW ratio	No. of mice
1	0.112	5	8.3	0.101	6
2	0.110	6	9	0.109	6
3	0.107	5	10	0.107	5
4	0.110	5	11	0.107	6
5	0.105	6	TSB (pH adjusted)	0.069	3
6	0.103	5	TSB (uninoculated)	0.066	3
7	0.108	6	Crude toxin (100°C)	0.095	6

[a] Preincubation was at 37°C for 4 h prior to neutralization and injection as described in Materials and Methods.

[b] GW/BW ratio in suckling mouse model assay for enterotoxin. Ratios of 0.083 or greater are considered positive.

ture supernatants until most of the glucose in the media had been utilized (Fig. 1). Although these findings suggest that glucose may also repress *Yersinia* enterotoxin synthesis, further studies using continuous culture techniques with control of pH and glucose content are needed to clarify the effects of these variables on *Yersinia* enterotoxin production.

When assayed in suckling mice, *Y. enterocolitica* enterotoxin is highly resistant to heating. We observed no reduction in enterotoxic activity after 30 min at 100 or 121°C, unlike *E. coli* ST, which is inactivated after 30 min at 121°C (12). *Yersinia* enterotoxin is also heat stable (100°C for 15 min) when assayed in the rabbit ileal loop assay (13). *Y. enterocolitica* enterotoxin and *E. coli* ST both resist low pH. Although enterotoxic activity of *E. coli* ST is not affected by acid, it is destroyed by exposure to pH 11 for 4 h at 37°C (5, 12). Under similar conditions, *Y. enterocolitica* A2627 crude toxin was unaffected by pH 1 through pH 11.

Kohler (8) and Mullan et al. (12) found that *E. coli* ST could be extracted from culture supernatants using methanol without losing its heat and pH stability and enterotoxic activity in suckling mice. In studying the similarities between *E. coli* ST and *Y. enterocolitica* enterotoxin, we felt that it was important to determine if *Yersinia* crude toxin was also methanol soluble. Our studies demonstrate that *Y. enterocolitica* enterotoxin is also methanol soluble and that methanol treatment does not appreciably affect its heat stability or activity in suckling mice.

Recently, Burgess et al. (3) have suggested that *E. coli* may produce two types of ST: STa, which is methanol soluble, partially heat stable, positive in the suckling mouse and neonatal pig assays, and negative in the rabbit ileal loop assay; and STb, which is methanol insoluble, heat stable, positive in the rabbit ileal loop assay, and negative in the suckling mouse and neonatal pig assays. Thus, evaluation of methanol-soluble extracts of *Yersinia* broth cultures in animal models other than mice may be useful in further characterizing *Yersinia* enterotoxins.

TABLE 5. *Enterotoxic activity of ultrafiltration fractions of Y. enterocolitica and E. coli heat-stable toxins*

Strain	Filter	GW/BW ratio	
		Retentate	Ultrafiltrate
A2627	PM-10	0.124	0.071
	UM-10[a]	0.128	0.049
7031	PM-10	ND[b]	ND
	UM-10	0.068	0.064
E. coli P-263	PM-10	0.136	0.130
	UM-10	0.141	0.072

[a] All UM-10 retentates and ultrafiltrates were heated to 100°C for 30 min prior to enterotoxin assays.
[b] ND, Not done.

TABLE 6. *Enterotoxic activity of methanol-soluble and methanol-insoluble fractions of Y. enterocolitica heat-stable toxin*

Strain	GW/BW ratio	
	Methanol soluble	Methanol insoluble
A2627	0.154	0.067
Heated[a]	0.137	0.072
Autoclaved[b]	0.148	ND[c]
7031	0.064	0.066
Heated	0.072	0.068
Autoclaved	0.066	ND

[a] Toxin heated to 100°C for 20 min prior to assay in suckling mice.
[b] Autoclaved (122°C) for 30 min.
[c] ND, Not done.

The molecular weight of *Yersinia* enterotoxin has not been determined. However, the pH and heat stability of the toxin suggest that it is a low-molecular-weight substance. In our ultrafiltration experiments, *Yersinia* enterotoxin was retained by a PM-10 membrane, whereas *E. coli* ST passed through the same membrane, suggesting that *Yersinia* enterotoxin may be somewhat larger than *E. coli* ST. Both *E. coli* ST and *Yersinia* enterotoxin were retained by the UM-10 membrane (nominal cutoff, molecular weight 10,000). Failure of the *E. coli* ST (molecular weight 4,000 to 6,000) to pass through the UM-10 membrane implies binding of *E. coli* ST molecules to higher-molecular-weight medium components. Therefore, techniques other than ultrafiltration, such as column chromatography or gel electrophoresis, are needed to more accurately determine the molecular weight of *Yersinia* enterotoxin.

Y. enterocolitica enterotoxin and *E. coli* ST are both heat and pH stable, are methanol soluble, and cause fluid accumulation in the suckling mouse assay. Although *E. coli* ST undoubtedly represents a virulence property in diarrhea of humans and domestic animals (9, 19), the clinical significance of *Yersinia* enterotoxin has not been established. Since *Y. enterocolitica* does not produce enterotoxin at 37°C in vitro, documentation of its production in vivo will be important in assessing its role in *Yersinia*-associated diarrhea. The resistance of *Y. enterocolitica* enterotoxin to extremes of temperature

and pH suggest that it may be capable of causing toxigenic food poisoning. Preformed toxin should be able to withstand the temperatures used in food processing and storage and presumably would not be inactivated by gastric acidity. Further studies are needed to determine if *Y. enterocolitica* is capable of elaborating enterotoxin in foodstuffs or dairy products.

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