INFECTION AND IMMUNITY, Aug. 1976, p. 383-388
Vol. 14, No. 2

Printed in U.S.A.

Factors Influencing Heat-Labile *Escherichia coli* Enterotoxin Activity

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Received for publication 30 December 1975

In this study, conditions for production, detection, and storage of heat-labile *Escherichia coli* enterotoxin (LT) in culture filtrates from *E. coli* H-10407 were defined by using the adrenal tumor cell assay system. An enriched medium containing 0.6% yeast extract, 2% Casamino Acids, and 0.25% glucose buffered at pH 8.5 produced the highest LT activity of the various test media. In *E. coli* strain H-10407, LT activity was markedly decreased if the initial pH of the culture media was reduced to pH 7.5 or less. In contrast to *E. coli* P-263, if strain H-10407 was grown in the presence of mitomycin C there was no increase in LT production. Crude-culture filtrates containing LT can be stored at 4°C for several days without an appreciable loss of activity; however, for long-term storage lyophilization or freezing at −70°C is recommended.

The development of a simple, reliable, and practical assay for enterotoxigenic *Escherichia coli* is necessary for epidemiological studies of diarrheal diseases and for routine evaluation of endemic diarrhea. The ileal loop assay for *E. coli* enterotoxin is time consuming, expensive, and not readily adapted to the routine diagnostic laboratory (1, 17). Cultured adrenal tumor cells as well as other established tissue culture lines such as Chinese hamster ovary cells, intestinal epithelial cells, and rat pituitary cells are now being used to study the role of heat-labile *E. coli* enterotoxin (LT) in diarrheal disease, to purify LT, and to investigate the mechanism of LT activation of adenylate cyclase (2, 6, 9-12, 15-19).

The ability of the adrenal cell assay to detect *E. coli* isolates producing low levels of LT would be increased if the optimal conditions for production and storage of LT in crude-culture filtrates were defined. In the past, various media, including Syncase, because of its success in cholera enterotoxin production, Trypticase soy broth, a yeast-supplemented semisynthetic media, and a defined minimal media, have been used to grow enterotoxigenic *E. coli* (2-4, 8, 14). In this study, the culture conditions influencing LT production and stability in a human enterotoxigenic strain, *E. coli* H-10407, were examined, using the sensitive, quantitative adrenal cell assay.

MATERIALS AND METHODS

Bacterial culture procedures. *E. coli* strain H-10407 (serotype O78:H11), strongly enterotoxigenic by the adrenal tumor cell assay, was kindly provided by S. Gorbach, Tufts-New England Medical Center, Boston, Mass. *E. coli* P-263 was kindly provided by H. Moon, National Animal Disease Center, Ames, Iowa. The stock cultures were maintained at room temperature on peptone slants composed of 2.0% peptone (Difco Laboratories, Detroit, Mich.), 0.5% NaCl, and 2.0% agar. There has been no loss of enterotoxigenicity for more than 2 years under these conditions provided *E. coli* H-10407 is periodically subcultured.

Initially, LT production in the following broth media was compared: (i) minimal salts medium [0.4% $NaHPO_4$, 0.02% KH_2PO_4, 0.05% $(NH_4)_2SO_4$, 0.05% $MgSO_4$, 0.25% glucose]; (ii) tryptone-glucose-yeast extract (1.0% tryptone [Difco], 0.5% yeast extract [Difco], 0.25% K_2HPO_4, 0.25% glucose); (iii) Casamino Acids-yeast extract-salts medium (CAYE) (2.0% Casamino Acids [Difco], 0.15% yeast extract [Difco], 0.25% NaCl, 0.871% K_2HPO_4, 0.25% glucose, 0.1% [vol/vol] trace salts solution consisting of 5% $MgSO_4$, 0.5% $MnCl_2$, and 0.5% $FeCl_3$); and (iv) Trypticase soy broth (TSB) (BBL, Cockeysville, Md.). The test media were all adjusted to a final pH of 8.5 with 1 N NaOH or 1 N HCl and sterilized by autoclaving for 15 min at 120°C. The glucose was sterilized separately and then aseptically added to the media except in the case of TSB which already contains 0.25% glucose. In subsequent experiments, CAYE medium with 0.6% yeast extract (CAYE-2) was used as the standard medium. In the experiment evaluating the role of high-molecular-weight material in enterotoxin production, CAYE-2 medium was passed through a TCF10 (Amicon, Lexington, Mass.) ultrafiltration system containing an XM-100A membrane filter.

Enterotoxin production and cell growth. Culture filtrates were prepared by growing *E. coli* H-10407 overnight in nutrient broth at 37°C and then adding a 0.5-ml inoculum to 20 ml of medium in a 125-ml Erlenmeyer flask. The cultures were agitated on a

rotary incubator at 130 rpm for 20 to 24 h at 37°C. The cells were removed by centrifugation at 12,000 × *g* for 15 min at 4°C, and the supernatant was filtered through a 0.22-μm membrane filter (Millipore Corp., Bedford, Mass.) before use in the tissue culture system. Culture filtrates were stored at 4°C, and generally a 1:10 dilution of filtrate was assayed within 1 to 2 days.

In the mitomycin C (Calbiochem, La Jolla, Calif.) experiments, the inhibitor was added to *E. coli* H-10407 or P-263 cultures 2 h after inoculation of CAYE-2. After 24 h of incubation, the supernatants were tested in the tissue culture system as previously described. A 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320 dilution of culture filtrate from mitomycin C-treated or control cultures was added to duplicate confluent adrenal cell cultures.

Bacterial cell growth was monitored by measuring the optical density of a 1:10 dilution of culture at 640 nm with a spectrophotometer (Bausch & Lomb, Rochester, N.Y.). When comparing growth in different types of media, the respective medium was used as diluent and reference blank.

Adrenal cell procedures. Y-1 mouse adrenal cortex tumor cells were maintained in monolayer cultures in Eagle minimal essential medium with Earle salts (MEM) (GIBCO, Grand Island, N.Y.) supplemented with 12.5% horse serum, 2.5% fetal calf serum, and 1.0 mM L-glutamine with penicillin (10 U/ml) and streptomycin (10 μg/ml). Cells were grown in petri dishes (60 by 15 mm) (Falcon Plastics) at 37°C in a humidified atmosphere of 5% CO_2 in air. Confluent monolayer cultures were washed with 2.0 ml of saline, then the desired dilution of culture filtrate in 2.0 ml of MEM was added for 20 h of incubation, and at the end of the incubation the medium was assayed for steroid content by a previously described method (18). The cell monolayer was washed with saline and lysed with 2.0 ml of 0.1 N NaOH, and the cell protein was determined by the method of Lowry et al. (13). The percentage of rounded cells per culture plate was estimated by using a phase-contrast microscope (Nikon MS) by determining the percentage of rounded cells in several representative fields.

pH stability and thermostability of LT. To study the effect of pH on LT, 2.0-ml portions of CAYE-2 culture filtrate were adjusted from pH 3 to 11 with 0.4 N NaOH or 0.4 N HCl. The acid or base was added in very small increments with rapid mixing as a precaution against toxin inactivation. After pH adjustment, the samples were sterilized by passage through a 0.45-μm membrane filter (Millipore) and incubated at room temperature for 5 h. Two-tenths-milliliter portions were then added to confluent adrenal monolayers overlayed with 2.0 ml of MEM. It was unnecessary to reneutralize the filtrates since, under these assay conditions, the final pH of the MEM was only minimally altered.

To determine the effect of temperature on toxin stability, three sets of samples were heated to 37, 60, and 100°C, respectively. A sample from each set was removed at 5, 15, and 30 min, brought to 4°C, and then assayed.

In studying temperature effects during long-term storage, portions were stored at 23, 4, −20, and −70°C, and activity was assayed at regular intervals for a period of 23 days. Portions were thawed only once before assaying steroidogenic activity.

RESULTS

Growth media and LT production. The effect of five types of media on the growth and the LT production of a human enterotoxigenic *E. coli* strain H-10407 was determined by using the cultured adrenal cell system (Table 1). Under these conditions, cell growth was greatest in the CAYE and CAYE-2 media. The total LT activity as assayed by alteration of adrenal morphology from flattened to spherical-shaped cells, and steroidogenesis was greatest in CAYE-2 medium. In minimal salts medium, a 1:10 dilution of culture filtrate produced no detectable change in steroidogenesis; in TSB, steroid production was increased fivefold; but in CAYE-2, steroidogenesis was increased 10-fold.

The effect of various concentrations of Casamino Acids and yeast extract on LT activity was studied (Table 2). The concentration of yeast extract was varied from 0 to 2% with a 2% Casamino Acids concentration, and then the Casamino Acids concentration was varied from 0 to 4% with a constant yeast extract concentration (0.15%). The results show that LT activity was greatest with a 0.6 or 0.8% yeast extract concentration and a 2% Casamino Acids concentration. Steroidogenic activity was decreased when the yeast extract concentration was 2% or when the Casamino Acid concentration was greater than 2%. Further studies showed that the optimal glucose concentration

TABLE 1. *Enterotoxin production in various growth media*

Growth medium[a]	OD[b]	Adrenal tumor cell assay	
		% of cells rounded	Steroid production[c]
MM	0.145	5	1.09
TGYE	0.440	40–50	4.74
TSB	0.450	50	5.13
CAYE	0.600	70–80	9.32
CAYE-2	0.600	70–80	10.04

[a] MM, Minimal salts medium; TGYE, tryptone-glucose-yeast extract; TSB, Trypticase soy broth; CAYE, Casamino Acids-0.15% yeast extract-salts medium; CAYE-2, Casamino Acids-0.60% yeast extract-salts medium.

[b] OD, Optical density of a 1:10 dilution of culture at 640 nm.

[c] Steroid production in response to a 1:10 dilution of culture filtrate is expressed as fold increase from the basal control level.

TABLE 2. *Effect of concentration of yeast extract (A) and Casamino Acids (B) on enterotoxin production and activity in CAYE medium*

Yeast extract				Casamino Acids			
% yeast extract in 2% Casamino Acids-salts medium	Cell growth[a]	Final pH of culture	Steroid production[c]	% Casamino Acids in 0.15% yeast extract-salts medium	Cell growth[a]	Final pH of culture	Steroid production
0	0.55	8.2	0.012	0	0.23	7.5	0.090
0.2	0.47	8.2	0.143	1.0	0.52	8.3	0.084
0.6	0.49	8.3	0.205	1.5	0.56	8.4	0.181
0.8	0.52	8.3	0.222	2.0	0.52	8.3	0.194
2.0	0.49	8.2	0.123	3.0	0.45	8.3	0.122
				4.0	0.29	8.3	0.119

[a] Optical density at 640 nm of a 1:10 dilution of culture.

[b] Steroid production is expressed as microgram per milligram of cell protein per hour and represents the increase over the basal level.

for LT activity was 0.25 to 0.5%. To determine the effect of components of CAYE-2 medium with a molecular weight greater than approximately 100,000 on steroidogenesis, the medium was filtered through an Amicon XM-100A membrane filter before inoculation with the enterotoxigenic strain. Under these conditions there was a one-third reduction in cell growth and LT production.

Effect of initial pH and aeration on LT. *E. coli* H-10407 was grown for 48 h in CAYE-2 medium adjusted to an initial pH of 6.5, 7.5, or 8.5 in order to evaluate the effect of pH on steroidogenesis (Fig. 1). It is clear that at pH 8.5 there was a marked increase in the steroidogenic activity of crude *E. coli* culture filtrates compared to cultures grown at an initial pH of 7.5. Under these assay conditions, increased steroidogenesis could be detected at 4 h of incubation and LT activity reached a maximum at 24 to 30 h of incubation. Further studies showed that if CAYE-2 was initially adjusted to pH 9, bacterial growth was markedly diminished. Figure 2 demonstrates the relationship between cell growth, pH changes, and toxin production at pH 6.5, 7.5, and 8.5. The pH of the cultures decreased significantly during the first 12 h of growth but returned to the initial levels by 18 to 24 h. Despite the differences in steroidogenic activity under the three culture conditions, there was no significant differences in cell growth.

Generally, there was a close correlation between the percentage of rounded cells and steroid production. Maximum cell rounding occurred by 22 h of incubation. Previously, alteration of adrenal monolayer morphology has been related to elevation of intracellular adenosine 3',5'-cyclic monophosphate levels (12). A comparison between agitated and stationary cultures showed that after 8 h of growth, the agitated culture contained two times the cells and LT activity as the stationary cultures. After 24 h of incubation, these differences were reduced.

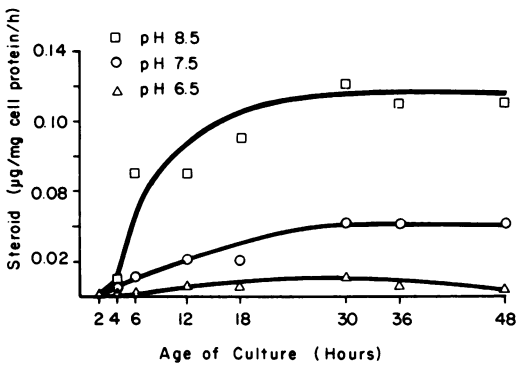

FIG. 1. *Effect of the initial pH of CAYE-2 media on LT production from crude culture filtrates.*

Effect of mitomycin C on LT. In certain enterotoxigenic strains of *E. coli*, growth in the presence of mitomycin C increases LT production as measured by the adrenal assay system (7). In *E. coli* H-10407, however, there was a significant decrease in the steroidogenic activity of culture filtrates treated with mitomycin C (Fig. 3). A 1:80 dilution of the control culture filtrate produced half-maximal steroidogenesis, and a 1:160 dilution caused rounding of 50% of the adrenal cells. In contrast, when this strain was grown in mitomycin C-supplemented medium, half-maximal steroidogenesis occurred at a 1:30 dilution of culture filtrate and at a 1:160 dilution only 10% of the cells were rounded. In a porcine strain, *E. coli* P-263, a 1:80 dilution of control culture filtrate and a 1:320 dilution of culture filtrate from mitomycin C-treated *E. coli* stimulated half-maximal steroidogenesis. When a correction was made for the marked reduction in cell growth in media supplemented

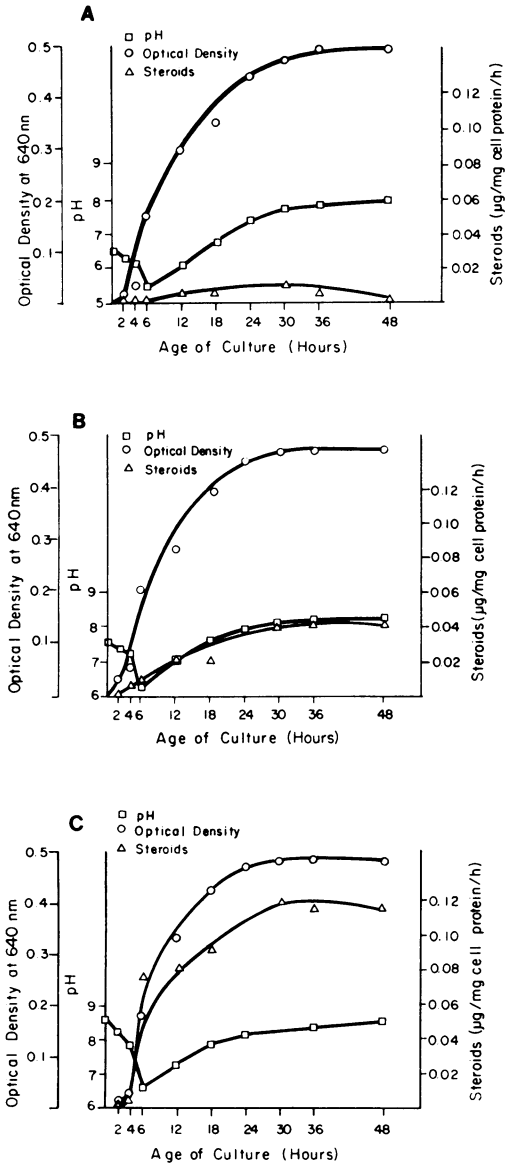

FIG. 2. *Relationship between cell growth, LT production, and pH changes in CAYE-2 medium. Initial pH values: (A) 6.5; (B) 7.5; and (C) 8.5.*

with mitomycin C, then the LT activity in the porcine strain was increased 10-fold by the addition of mitomycin C.

Effect of pH, temperature, and storage on LT activity. If culture filtrates containing LT were adjusted from pH 6 to pH 11 and incubated at 22°C for 5 h, there was no loss of steroidogenic activity. However, below pH 6 there was marked loss of LT under these incubation conditions. In addition, activity in the adrenal cell assay was reduced by one-third when culture filtrates were heated at 60°C for 5 min (Table 3). More than 90% of the steroidogenic activity of the culture filtrates was lost after heating at 60 C for 15 min.

Table 4 shows the effect of storage at various temperatures on steroidogenic activity of culture filtrates. Initially, there was a small loss of activity at −20 and −70°C. After 16 days of

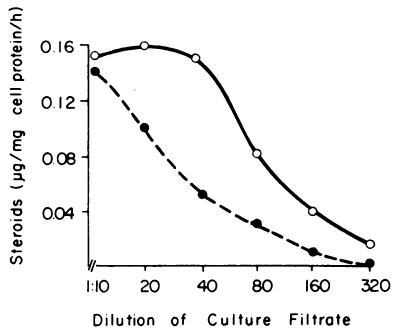

FIG. 3. *The steroidogenic response of adrenal cells to various dilutions of E. coli H-10407 culture filtrate after mitomycin C treatment (●) compared to control cultures (○).*

TABLE 3. *Thermal stability of LT in crude-culture filtrate*

Time of heating (min)	Steroidogenic activity[a] at:		
	37°C	60°C	100°C
0 (Control at 4°C)	0.046	0.046	0.046
5	0.039	0.029	0
15	0.046	0.004	0
30	0.050	0.001	0

[a] Steroid activity is expressed as micrograms per milligram of cell protein per hour and represents the increase over the basal level.

TABLE 4. *Effect of storage temperature on LT activity*

Length of storage (days)	Steroidogenic activity[a] at:			
	23°C	4°C	−20°C	−70°C
3	0.040	0.051	0.038	0.033
16	0.022	0.023	0.005	0.022
23	0.002	0.008	0.001	0.023

[a] Steroidogenic activity is expressed as micrograms of steroids produced per milligram of cell protein per hour and represents the increase over the basal level. Because steroid production by different sets of cells varied, the activity values for a particular temperature for the different days shown are not entirely comparable. However, the activity values are quantitatively comparable for the different temperatures for a given day.

storage, toxin activity at 23, 4, and −70°C were comparable, whereas there was a significant loss of activity at −20°C. However, after 23 days of storage at 4°C, LT activity was less than one-third of the activity of the culture filtrate stored at −70°C, and the −20°C sample had almost no activity. This experiment demonstrates that crude culture filtrates from *E. coli* H-10407 can be stored at −70°C for more than 3 weeks without an appreciable loss of steroidogenic activity.

DISCUSSION

The effect of various culture and storage conditions on LT activity, as measured in cultured adrenal cells, was investigated in order to define the optimal conditions for detecting LT in cell-free culture filtrates from suspected enterotoxigenic strains of *E. coli*. Although adrenal cells are very sensitive to LT, it is possible that LT activity varies among strains and therefore optimal assay conditions for the adrenal system should be employed.

Evans et al. (4) have investigated various factors that are favorable to the production of LT by use of the rabbit skin (PF) and ileal loop assays. As a result, these workers employ a well-buffered Casamino Acids-yeast extract-salts growth medium. They were able to show that yeast extract of a minimal concentration of approximately 0.02% was necessary for detection of PF activity in crude culture filtrates, and suggest that other media commonly used for cholera enterotoxin production do not meet this minimal requirement. In the present study it was found that yields of LT were consistently higher in CAYE medium compared to minimal salts medium, tryptone-glucose-yeast extract, and TSB media. Our results show that LT activity was enhanced by increasing the yeast extract concentration to 0.6 or 0.8%, in the presence of 2% Casamino Acids and 0.25% glucose. Carbohydrate was omitted from the medium used by Evans et al. in order to eliminate acid inactivation of LT (4). These workers suggest that the presence of this component, especially in weakly buffered media, causes a decrease in pH that may lower the yield of LT. In this study, we found that although there was an initial decrease in pH, glucose supplementation of CAYE medium enhanced the overall yield of LT at 24 h of incubation. However, glucose concentrations above 0.5% caused a definite decline in steroidogenic activity, presumably because of greater acid production.

A recent report describes the use of a defined medium containing salts, glucose, and vitamins for the production and purification of porcine LT (14). Since in LT purification studies it may be advantageous to start with preparations containing as little interfering proteins as possible, the effect of elimination of large-molecular-weight substances from CAYE-2 medium was studied. The removal of material of greater than 100,000 molecular weight led to a significant decrease in cell growth and LT production. This result suggests that in CAYE-2 high-molecular-weight substances are essential for maximal LT production. In purification studies, however, it may be preferable to use a synthetic culture medium in order to eliminate extraneous contaminating proteins.

The pH of the growth medium throughout the incubation period had a profound effect on toxin activity. Our results show that after 24 h of growth LT activity was almost doubled if the initial pH of CAYE-2 was 8.5 rather than 7.5. Steroidogenic activity was minimal if the initial pH of CAYE-2 was 6.5. The differences in LT yield suggest that a substantial amount of released LT is inactivated when the pH of the medium decreases, which occurs concomitantly with glucose utilization and acid production. The pH decreased slightly below pH 7 for only a brief period in pH 8.5 medium; therefore, minimal LT inactivation occurred under this condition. Cell growth at the three starting pH levels was approximately equal, indicating that the differences in LT activity were independent of growth.

In this study we confirmed the inducibility of LT in *E. coli* P-263 reported by Isaacson and Moon (7). In that study, six *E. coli* strains producing LT were induced by mitomycin from 8- to 96-fold. However, in the human enterotoxigenic strain *E. coli* H-10407, mitomycin C did not induce LT production. This human strain contains several extrachromosomal genetic elements in addition to the enterotoxigenic plasmid (W. Maas, personal communication), and this may be related to the inability of mitomycin C to induce LT production.

Our results indicate that the pH of culture filtrates is as important as growth conditions for maintaining LT activity. The adrenal assay results show that LT activity in culture filtrates is stable from pH 6 to pH 11 when incubated at room temperature for 5 h. Jacks et al. reported a pH optimum of 7.0 for partially purified LT from an *E. coli* strain enteropathogenic for swine (8). The effect of storage on culture filtrate activity showed that LT was stable at 4°C for at least 3 days but at 16 days activity was reduced by approximately 50%. It is important to emphasize that LT was least stable at −20°C. To retain LT activity for more than 3 weeks, culture filtrates can be rapidly frozen and stored at −70°C or lyophilized (5).

ACKNOWLEDGMENTS

This investigation was conducted by D.H.M. during his graduate studies completed at California State University, Long Beach, Calif. in June 1975. This work was supported by Public Health Service grant CA 16868 from the National Cancer Institute and by the Medical Research Programs, Veterans Administration Hospital, Long Beach, Calif.

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