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Induction of Heat-Labile Enterotoxin Synthesis in Enterotoxigenic *Escherichia coli* by Mitomycin C

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Induction of heat-labile toxin (LT) synthesis in enterotoxigenic strains of *Escherichia coli* by mitomycin C (MTC) was demonstrated. Six enteropathogenic strains which produce LT were inducible, exhibiting an 896-fold increase in LT when compared to uninduced cultures. On the other hand, four nonenteropathogens and three other pathogens which produce only the heat-stable toxin were not induced to produce LT. Gel filtration chromatography, antibody neutralization, and heat lability studies suggest that the toxin synthesized in the presence of MTC is the same as the toxin synthesized in the absence of MTC.

Enterotoxigenic plasmids (Ent) are nonessential extrachromosomal genetic elements found in some enteropathogenic strains of *Escherichia coli* (15, 16). The presence of an Ent plasmid confers on the host strain the ability to elaborate a diarrhea-inducing enterotoxin(s). Two classes of Ent plasmids have been identified. One Ent plasmid presumably codes for the synthesis of a heat-labile toxin (LT) and a heat-stable toxin (ST). The second type of Ent plasmid codes only for ST (14).

Studies on the interaction between toxins and target site have been hampered by the lack of an adequately pure material. It is clear that any genetic manipulation that may be performed to increase the amount of toxin synthesized would be beneficial to the overall design of purification schemeta and other studies employing LT. Various agents have been previously demonstrated to be useful in the derepression of some plasmid-related functions. For example, ultraviolet light irradiation (12), treatment with antibiotics, including mitomycin C (MTC) (5, 6, 7), chloramphenicol (1, 9), or puromycin (1), and thymine starvation (13) are useful inducing agents for various colicin genes. In such cases, there is a large increase in the amount of colicin synthesized after treatment.

This study was undertaken to determine whether heat-labile toxin synthesis could be increased by treatment of enteropathogenic *E. coli* with MTC. The term induction is used to indicate enhanced toxin synthesis. In addition, the size of the toxin was investigated before and after such treatment to determine whether gene derepression results in the synthesis of a toxin of the same or different size.

MATERIALS AND METHODS

Organisms. The various strains of *E. coli* are described in Table 1. All strains except 1347 and 1348 were originally isolated from pigs. Strain 1347 was isolated from chickens. Strain 1348 was constructed by transfer of an LT-ST plasmid to strain 1347.

Media and preparation of toxins. Stationary-phase bacteria were inoculated 1:50 into Trypticase soy broth (BBL) and incubated at 37 C with vigorous shaking. When required, MTC (Sigma) was added 2 h postinoculation. After 18 h of incubation, the cells were harvested by centrifugation at 12,000 × *g* for 10 min at 4 C. The cells were discarded, and the supernatant fluid was filtered through sterile 0.45-μm nitrocellulose filters (Millipore Corp.) and stored at 4 C for future use. The supernatant fluid served as the toxin for further experiments unless otherwise specified. When stored in the cold, the toxin lost no activity (assayed as described below) over a 4-month period. When necessary, the toxin was concentrated by centrifugation in a Spinco type 30 rotor at 30,000 rpm for 18 h at 4 C. The resultant pellet was gently resuspended in an appropriate amount of 0.05 M tris(hydroxymethyl)aminomethane (Tris), pH 8.0.

Tissue culture assay for heat-labile toxin. Labile toxin was assayed on Y-1 mouse adrenal tumor cells (2). Adrenal cells were grown in 16-mm tissue culture plates (Limbro Chemical) containing 1 ml of medium (Ham's F10 containing 20% fetal calf serum and 50 μg of gentamicin [Schering] per ml) at 37 C in a humidified atmosphere of 95% air and 5% CO_2. Toxin preparations were diluted serially twofold in 0.01 M Tris, pH 8.0. When a monolayer of adrenal cells had grown, 0.1 of toxin was added, and the plate was incubated for an additional 20 h. Cell rounding, an indicator of LT activity (2), was observed by microscopic examination of test plates. The number of units of toxin activity was defined as the reciprocal of the highest dilution causing adrenal cell rounding.

RESULTS

Induction of LT synthesis by MTC. Strain 263, an LT-ST producer, was grown for 18 h in the presence of various amounts of MTC. The addition of MTC causes a marked increase in

the amount of LT activity (Fig. 1). The addition of as little as 0.1 μg of MTC per ml of culture causes maximal stimulation. The increase in LT activity was 12-fold in this experiment; however, in other similar experiments using strain 263, close to a 100-fold increase in LT activity was observed using 0.5 μg of MTC per ml. Similar increases in LT activity were also observed in response to MTC when LT was assayed from bacterial cell lysates (sonication for 90 s). Since the toxin preparations used in these experiments were the broth culture supernatants, a substantial quantity of MTC should be present. Therefore, it was necessary to determine whether Y-1 adrenal cells would respond to MTC in a manner similar to LT. When 0.5, 1.0, or 10 μg of MTC per ml was added to adrenal cell test plates and incubated as described in Materials and Methods, no cell rounding was ever observed. A second reason why an increase in LT activity was observed might be that this was a result of activation of pre-existing toxin, or a "protoxin," by MTC. Toxins prepared from uninduced (i.e., not treated with MTC) cultures were mixed with 0.5 μg of MTC per ml, incubated 4 or 18 h at 37 C, and assayed for toxin activity. No increase in LT activity was observed. In addition, when a whole cell lysate was prepared by sonication for 90 s, mixed with 0.5 μg of MTC per ml incubated for 4 or 18 h at 37 C, and assayed for toxin activity, no increase in LT activity was measured.

The requirement for de novo protein synthesis for the induction of LT synthesis is shown in Fig. 2. It can be seen that the addition of chloramphenicol to broth cultures of strain 263 inhibits the increase in toxin activity when compared to untreated or MTC-treated cultures. This effect occurs irrespective of the addition of MTC. When chloramphenicol (200 μg/ml) was added to adrenal cells (no toxin added), no cell rounding was observed. If chloramphenicol was mixed with preformed toxin, no decrease in toxin activity occurred. These experiments clearly demonstrate the requirement of protein synthesis to see enhanced LT activity. This probably reflects synthesis of new toxin molecules.

TABLE 1. *E. coli strains used*

Strain	Serotype	Toxin produced	Source
123	O43:K⁻		
124	O8:K50		
252	O13:K⁻		
1347	O18ab:K(?)		C. L. Gyles, F11
1348	O18ab:K(?)	LT-ST	C. L. Gyles, F11 (P155)
263	O8:K87, 88ab	LT-ST	
1362	O149:K88ac	LT-ST	R. Ellis, 72-2502
1260	O141:K85ac, 88ab	LT-ST	D. A. Barnum, G1108E
1288	O149:K91, 88ac	LT-ST	C. L. Gyles, PA1
1291	O149:K91, 88	LT-ST	
431	O101:K30	ST	
987	O9:K(A)	ST	
1261	O138:K81	ST	D. A. Barnum, 2176E8

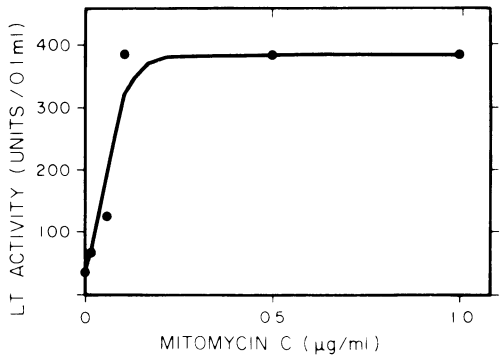

FIG. 1. *MTC induction of LT synthesis in strain 263. Cells were grown with the indicated amounts of MTC as described in Materials and Methods. Toxins were prepared and assayed as described in Materials and Methods.*

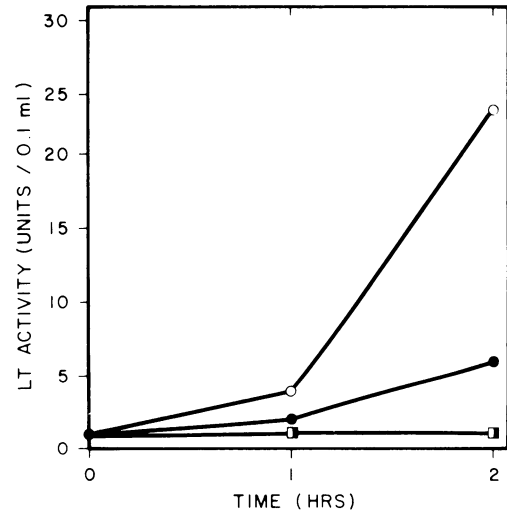

FIG. 2. *A culture of 263 was grown to a density of approximately 2 × 10⁸ cells/ml and split into four equal portions. One portion was left untreated (●). To the second portion chloramphenicol (200 μg/ml) was added (■). To the third portion MTC (0.5 μg/ml) was added (○). To the fourth portion MTC and chloramphenicol were added (□). These cultures were incubated at 37 C with shaking. At the indicated times, samples were removed and assayed for LT activity.*

Various other *E. coli* strains described in Table 1 were tested for MTC inducibility of toxin synthesis. Included in this group were LT-ST strains, strains producing ST, and several nonenteropathogenic strains. The results are summarized in Table 2. All LT-ST strains were inducible for LT synthesis. The minimum stimulation observed was eightfold and the maximum was 96-fold. On the other hand, no LT activity was detected in toxin preparations from ST-only strains or non-enteropathogenic strains, irrespective of MTC addition.

Toxin preparations showing activity in Table 2 were tested for heat lability and antibody neutralizability. All were inactivated when heated to 100 C for 15 min. Also, these same preparations were neutralized by antisera from pigs prepared against toxins from strains 263 or 1362, but not by normal pig serum. The results are summarized in Table 2. These two criteria are characteristically used to identify labile toxin (4). It therefore appears as if the adrenal cell-activating material synthesized after MTC addition is LT.

Molecular weight distribution of labile toxin. It was considered possible that toxin synthesized in the presence of MTC might be of a different molecular weight than the toxin synthesized in the absence of MTC. Therefore, a series of comparative size studies was performed using gel filtration chromatography.

TABLE 2. *Induction of heat-labile enterotoxin synthesis by mitomycin C*

Strain	LT activity (U/0.1 ml)			Heat inactivation[b]	Antibody neutralization[c]
	−MTC	+MTC[a]	+MTC/−MTC		
123	<1	<1			
124	<1	<1			
252	<1	<1			
1347	<1	<1			
1348	<1	24	>24	Yes	Yes
263	3	256	85	Yes	Yes
1362	2	16	8	Yes	Yes
1260	0.5	48	96	Yes	Yes
1288	8	384	48	Yes	Yes
1291	2	96	48	Yes	Yes
431	<1	<1			
987	<1	<1			
1261	<1	<1			

[a] Cultures (0.5 μg of MTC per ml) were grown as in Materials and Methods.

[b] Undiluted samples were heated to 100 C for 15 min.

[c] Undiluted samples were mixed with a 1:50 dilution of serum, incubated for 30 min at 37 C, and assayed for toxin activity as described in Materials and Methods.

Toxin samples were first concentrated. Centrifugation of equal volumes of toxin produced by strain 263 resulted in a small translucent pellet when no MTC was included in the growth medium and a much larger, whitish pellet when MTC was included. The pellet from the MTC-induced culture contained 12.8 times more toxin activity and 12.4 times more absorbance at 280 nm (A_{280}). Prior to concentration, the MTC-induced culture showed 12.2 times more toxin activity (see Table 3). The concentrated materials were then resuspended and chromatographed through a column of Bio Gel A15 (exclusion size, 15×10^6), which had been equilibrated and subsequently eluted with 0.05 M Tris, pH 8.0. The resultant elution profiles are shown in Fig. 3. It can be seen that the toxin activity elutes at the same rate irrespective of MTC (fraction 1230 with MTC and fraction 1731 without MTC). The location of LT activity, in both cases, also correlates very well with the location of rapidly migrating A_{280} absorbing material. It should be pointed out that five times more sample volume was applied to the column in the case of uninduced 263. This was done to facilitate detection of the toxin. If the appropriate corrections are made, there is 13.8 times more toxin activity eluting in the toxin peak when MTC was included in the growth medium (results are summarized in Table 3) and 14.5 times more A_{280} absorbing material.

To eliminate the possibility that a substantial amount of lower-molecular-weight toxin was being lost in the centrifugation step, unconcentrated samples (±MTC) were applied to a Bio Gel A1.5 column (data not shown). No low-molecular-weight toxin was observed (i.e., all activity was in the column void volume). This does not exclude the possibility that a minor nondetectable amount (by adrenal cell assay) of lower-molecular-weight LT exists. However, it does say that the majority of LT is high molecular weight.

DISCUSSION

The data presented demonstrate the inducibility of heat-labile toxin synthesis in *E. coli*

TABLE 3. *Summary of LT activity from strain 263*

Step	LT activity (U/0.1 ml)		+MTC/−MTC
	−MTC	+MTC	
Broth supernatant	32	392	12.2
Centrifugation	128	1,540	12.8
A15 column	13[a]	180	13.8

[a] Activity corrected for amount applied to column.

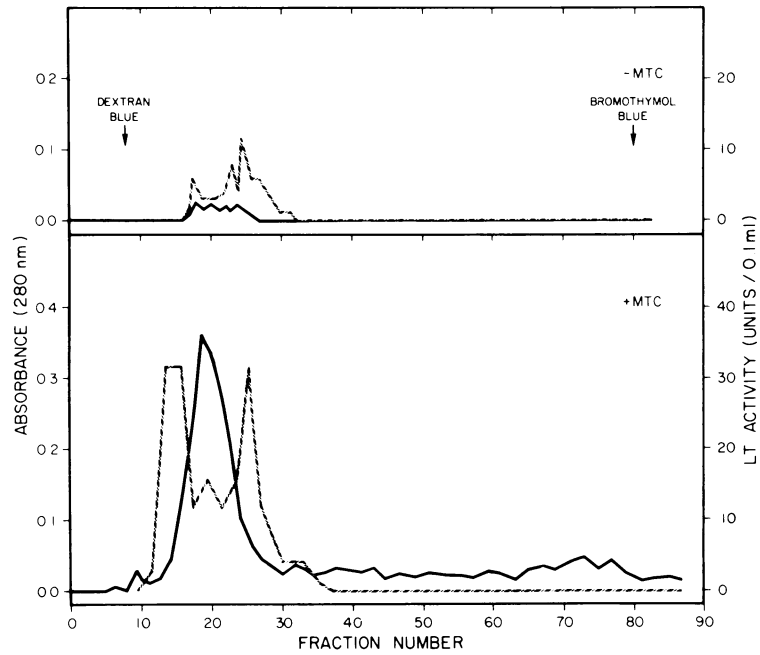

FIG. 3. *Gel filtration of heat-labile toxins from strain 263. A 5-ml portion of concentrated toxin from uninduced 263 or a 1-ml portion of concentrated toxin from induced 263 was applied to Bio Gel A15 (4 by 30 cm), equilibrated, and eluted with 0.05 M Tris, pH 8.0. Fractions of 2.8 ml were collected.* A_{280} *(solid line) was determined in a Beckman DU spectrophotometer, and LT (striped line) was assayed as described in Materials and Methods. Dextran blue and bromothymol blue were incubated to indicate the column excluded and included volumes, respectively.*

strains harboring the LT-ST Ent plasmid. All strains tested which were previously designated as LT-ST producers were inducible. Forsyth, using the rabbit ileal loop to assay LT, has also shown MTC inducibility of LT synthesis (personal communication). In addition, it has been shown that strains presumed to produce either no toxin or only ST cannot be stimulated to synthesize LT by addition of MTC. One can envision a situation whereby all *E. coli* strains are, under the appropriate conditions, capable of producing an LT. The lack of MTC inducibility of these strains, though not a rigorous proof, does tend to rule out this possibility. It is, of course, possible that another agent could stimulate LT synthesis in these strains. Preliminary data (unpublished) indicate that ST is not MTC inducible in ST only or in LT-ST strains.

The toxin size studies were performed to determine whether the LT molecule synthesized after MTC induction was the same size as the naturally synthesized molecule. It has been previously shown that the heat-labile enterotoxin is inactivated by pancreatic lipase (8, 11), as well as by several proteases (8, 11, 17). Jacks et al. (8) speculated that LT is a protein moiety embedded in a lipopolysaccharide backbone (possibly endotoxin). Based on this model, one would expect that under the appropriate conditions it should be possible to isolate a toxin molecule of lower molecular weight. In support of this hypothesis, Evans et al. (3) have recently isolated an LT with a molecular weight of 2×10^4 after treating enterotoxigenic *E. coli* with polymyxin B (LT typically seen has a molecular weight greater than 5×10^6 [8].) The evidence presented here indicates that under conditions of increased LT synthesis a smaller (2×10^4)-molecular-weight LT is not detected. Induction in LT synthesis by MTC results in a toxin which resembles the naturally synthesized LT.

The simplest interpretation of the phenomenon of LT inducibility is one of gene derepression. The majority of cells in an LT-ST-producing population are normally repressed with respect to LT synthesis. Only a few cells in the population are actively producing toxin at any given time. Addition of MTC probably causes a derepression of LT gene(s), causing a subsequent increase in LT synthesis. Similar regulatory phenomena have previously been demonstrated for colicins in colicinogenic populations (10). We are not at this time implying that the

molecular aspects involved in regulation of LT synthesis are the same as those for colicins. However, the identification of MTC inducibility of LT does give an excellent starting point for studying the molecular regulatory mechanisms involved in its synthesis.

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