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# Cellular Release of Heat-Labile Enterotoxin of *Escherichia coli* by Bacteriophage Induction

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Treatment of some enterotoxigenic *Escherichia coli* strains with the antibiotic mitomycin C resulted in lysis of the bacteria. Heat-labile enterotoxin (LT) activity of culture filtrates, determined by means of the Y-1 adrenal cell assay, increased dramatically as lysis of the culture proceeded. Further studies with *E. coli* strains 263 and B21-4 revealed that lysis is due to mitomycin C induction of vegetative development of a temperate bacteriophage. These findings suggest that the elevated levels of LT detected after mitomycin C treatment reflect the lytic release of cell-bound LT rather than the induction by mitomycin C of de novo toxin biosynthesis. Comparable increases in LT activity also resulted from thermal induction of a phage P1Cm lysogen of strain 263 or from sonic disruption of enterotoxigenic strains.

It is now well documented that some enteropathogenic *Escherichia coli* cause disease by colonizing the small bowel and producing enterotoxins that have been designated ST (heat-stable toxin) and LT (heat-labile toxin) (3, 4, 7, 14). The synthesis of these diarrhea-inducing enterotoxins by *E. coli* can be plasmid mediated (5, 15-17). Two fundamental classes of Ent plasmids have been recognized: one determines production of both LT and ST (3, 17), whereas a second type of Ent plasmid determines only ST (5, 17).

These enterotoxins can be readily detected, by use of both in vivo and in vitro assay systems, in cell-free supernatant fluid following growth of the bacteria in broth media. In addition, it has recently been reported by Isaacson and Moon (6) that the antiobiotic mitomycin C will induce the synthesis of LT by *E. coli*, presumably by a mechanism in which LT gene(s) are derepressed and de novo toxin synthesis is initiated. This conclusion was based on the finding that supernatant fluids from such mitomycin C-treated cultures yielded greater LT activity than untreated cultures (6).

Our present study reveals that an alternate mechanism can account for the increased LT levels following treatment of *E. coli* LT strains with mitomycin C. We have shown that this treatment results in lysis of the bacteria due to the induction of vegetative development of bacteriophages, which can lysogenize these enterotoxigenic *E. coli* strains. The increased levels of LT in filtrates of mitomycin C-induced strains thus may reflect the release of cell-bound LT during lysis rather than the induction of de novo toxin synthesis.

## MATERIALS AND METHODS

**Bacterial strains and bacteriophage P1Cm.** Most studies were performed with *E. coli* strain 263 (provided by H. Moon), which had been employed in the study of Isaacson and Moon (6). In preliminary screening studies we used a number of LT-producing *E. coli* strains associated with disease from diverse geographic areas. Strains TD427C1, TD504C1, TD286C2, and B21-4 were provided by R. Giannella; strains H10407, 334a, and B2C were part of the collection at Walter Reed.

For some studies with *E. coli* 263, we employed the thermoinducible phage P1Cm. This phage, provided by J. L. Rosner, is a natural recombinant between phage P1 and an R plasmid (2, 8, 12), which retains its capacity to lysogenize.

**Media.** Penassay broth (Difco) and Trypticase soy agar (BBL) were employed for routine cultivation of organisms. Penassay broth, although not optimal for synthesis of LT, was found to be superior for mitomycin C induction of bacteriophage synthesis. The amount of LT activity in Penassay broth cultures of *E. coli* 263 was comparable to the levels detected by Isaacson and Moon (6) in Trypticase soy broth cultures.

Trypticase soy agar containing chloramphenicol at 25 μg/ml was used for the isolation of P1Cm lysogens of *E. coli* 263.

**Assay of heat-labile enterotoxin.** LT was assayed on Y1 mouse adrenal cells by use of the technique of Sack and Sack (13) after slight modification. Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$, in Ham F-10 medium (Flow Laboratories) supplemented with 12.5% horse serum, 2.5% fetal calf serum, gentamicin (50 μg/ml), and penicillin G (100 U/ml). Approximately 4,000 cells suspended in 0.1 ml of medium were planted in each

of 96 wells of a microtiter plate. Cells were incubated for 72 h and washed, and 0.1 ml of fresh medium was added to each well. Then, 0.05 ml of sterile culture filtrate was added to each test well. After 18 to 24 h of incubation, cells were observed for rounding.

**Mitomycin C induction.** Bacteria from overnight stationary cultures were diluted 1:100 into fresh, prewarmed Penassay broth and incubated at 37°C with aeration. When growth had reached about $2 \times 10^8$ cells per ml (at an optical density at 550 nm [$OD_{550}$] of ~0.300), the culture was divided. Mitomycin C (Sigma Chemical Co.) was added to one portion at a final concentration of 1 μg/ml of culture. These cells and those in the control portion were further incubated with vigorous shaking at 37°C. After overnight incubation, the cells were removed by centrifugation at 10,000 × *g* for 20 min at 4°C. The cells were discarded and the supernatant was filter sterilized (0.45 μm, Millipore Corp.) and stored at 4°C. For kinetic experiments, samples were periodically removed and sterile filtrates were prepared as described above. Enterotoxin assays were performed on these supernatant filtrates usually within 24 h.

**Electron microscopic studies.** Preparations considered to contain phage or phagelike particles were examined in a JEOL 100-C electron microscope. For staining, a droplet of this preparation was placed on a grid (200 mesh copper with a Parlodion film) and mixed with a droplet of freshly prepared 1% phosphotungstic acid. After 10 min, the staining solution was removed by touching the grid to the corner of an absorbent filter paper. Grids with samples were examined shortly after staining at a magnification ×50,000. The exact magnification was determined by using a calibration grid made from a carbon grating. Dimensions were established by comparing measurements of particles with measurements of the calibration grid taken at the same magnification.

**Lysogenization of *E. coli* 263 with P1Cm.** A lysate of P1Cm (about $1 \times 10^9$ plaque-forming units/ml) was mixed with *E. coli* 263 at a multiplicity of five phages per cell. After incubation at 32°C for 2 h, the cells were spread on Trypticase soy agar plates containing 20 μg of chloramphenicol per ml and incubated for 48 h at 32°C. Lysogens of 263, directly selected by taking advantage of the chloramphenicol resistance determinant carried by this phage, were recovered at a frequency of about $2 \times 10^{-8}$ per plaque-forming unit. The presence of P1Cm in such lysogens was confirmed by thermal induction experiments (2, 12).

**Sonic oscillation procedures.** Strains to be lysed by sonic treatment were grown at 37°C in 30 ml of Penassay broth to an $OD_{550}$ of about 1.0. These cultures were chilled for 30 min at 4°C and then sonically disrupted with a Branson model LS 75 Sonifier equipped with a S75 probe. Sonic treatment was applied intermittently (10 s on, 10 s off) for the total times indicated, and the cultures were kept in an ice bath so as to minimize heat inactivation of LT. Sonicates were filter sterilized (0.45-μm membrane, Millipore Corp.) and assayed for LT.

## RESULTS

### Treatment of *E. coli* 263 and other LT strains with mitomycin C.

Preliminary studies with *E. coli* 263 yielded findings similar to those described by Isaacson and Moon (6) for this strain. After incubation overnight in the presence of mitomycin C, the enterotoxin activity of culture filtrates of 263 and other LT-producing *E. coli* strains was determined. As shown in Table 1, addition of mitomycin C to cultures of 263, B21-4, and TD286C2 caused a 32- to 80-fold increase in LT activity of sterile supernatant fluids. The remaining strains that were tested revealed slight or no increase in LT activity as compared with untreated control cultures.

A time course study of the effect of mitomycin C on *E. coli* 263 is presented in Fig. 1. At a density of about $2 \times 10^8$ cells per ml, an exponential culture was divided into two equal portions (50 ml). Mitomycin C was added to one portion, whereas the other served as a control. Cell mass ($OD_{550}$) and enterotoxin activity of sterile filtrates were determined for samples that were periodically removed.

When mitomycin C at a final concentration of 1.0 μg/ml was added to such a culture of strain 263, cell growth continued to increase for 30 to 45 min at a rate typical of the control culture. At this time, growth was affected and lysis of the culture was initiated, as shown by a leveling and subsequent decrease of OD. Moreover, the LT activity of culture filtrates increased dramatically (32-fold over control) as lysis of the culture proceeded. A similar lytic event was seen with strain B21-4. In contrast, *E. coli* H10407, which showed no increase in LT activity after mitomycin C treatment, failed to lyse even when 10 μg of mitomycin C per ml of culture was employed.

This course of events, which resulted in cell lysis, was suggestive of the classic phenomenon of mitomycin C induction of bacteriophage synthesis in lysogenic bacteria (9, 10). We thus

TABLE 1. *Enterotoxin (LT) activity after treatment with mitomycin C*[a]

| *E. coli* strain | LT activity (U/0.05 ml)[b] | | Increase in activity (+/−) |
|---|---|---|---|
| | −Mitomycin C | +Mitomycin C | |
| 263 | 8 | 640 | 80 |
| H10407 | 4 | 4 | |
| B21-4 | 10 | 320 | 32 |
| TD427C1 | 10 | 10 | |
| 334a | 10 | 20 | 2 |
| TD504C1 | 10 | 20 | 2 |
| B2C | 2 | 10 | 5 |
| TD286C2 | 2 | 160 | 80 |

[a] Treated with 1 μg of mitomycin C per ml.

[b] LT activity = reciprocal of highest dilution of filtrate causing Y-1 cell rounding.

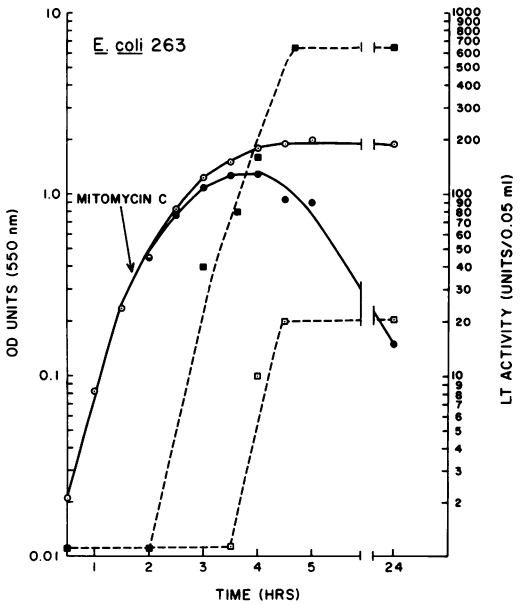



FIG. 1. *Induction of lysis of E. coli 263 by mitomycin C. Samples were removed and assayed for enterotoxin, as described. Symbols: Control (no mitomycin C): ⊙, $OD_{550}$; and ⊡, LT activity; mitomycin C added: ●, $OD_{550}$; and ■, LT activity.*

considered the possibility that some LT strains harbor temperate phages that respond to mitomycin C induction.

**Lysogency of *E. coli* 263.** The existence of a temperate phage lysogenizing *E. coli* 263 could not be demonstrated by usual biological tests because all efforts to identify a lytically sensitive host for such a phage failed. As a consequence, an electron microscopic search for a phage associated with *E. coli* 263 was undertaken by a procedure similar to that described by Parker et al. (11) for purification of phage-associated material from *Vibrio cholerae*. An exponential culture of *E. coli* 263 (100 ml) was induced to lysis by addition of mitomycin C (1 μg/ml). After lysis was complete, cells and other debris were removed by low-speed centrifugation (5,000 × *g*, 15 min, 4°C). This supernatant was next centrifuged at 35,000 × *g* (60 min, 4°C), and the pellet was suspended in 1.0 ml of distilled water. Preliminary electron microscopic examination revealed that this crude preparation contained uniform phage-like particles. As a consequence, this preparation was purified further by means of CsCl equilibrium density gradient centrifugation. Spectrophotometric analysis of the fractions obtained from density gradient centrifugation of this purified 263 lysate revealed a single homogeneous band of material (Fig. 2). The material in this fraction was dialyzed against 0.1 M ammonium acetate (pH 7.0) and then examined in the electron microscope.

As shown in Fig. 3, typical phage particles were present in mitomycin C-induced lysates of *E. coli* 263. This phage, designated φ263, has an isometric head with hexagonal symmetry and a short tail with no detectable contractile sheath. The phage particles appear uniform in size, with a head diameter of about 65 nm and a tail assembly of about 13 nm in width and 15 nm in length. The tail consists of three or more spikes and may contain a plate at the distal end.

*E. coli* B21-4, which also lysed upon addition of mitomycin C, likewise was found to be lyso-

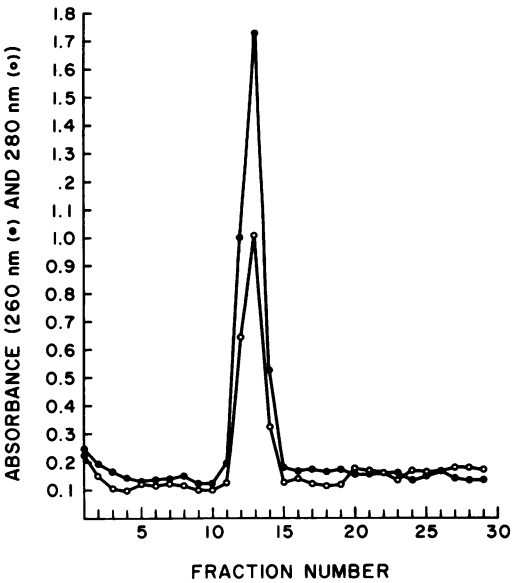
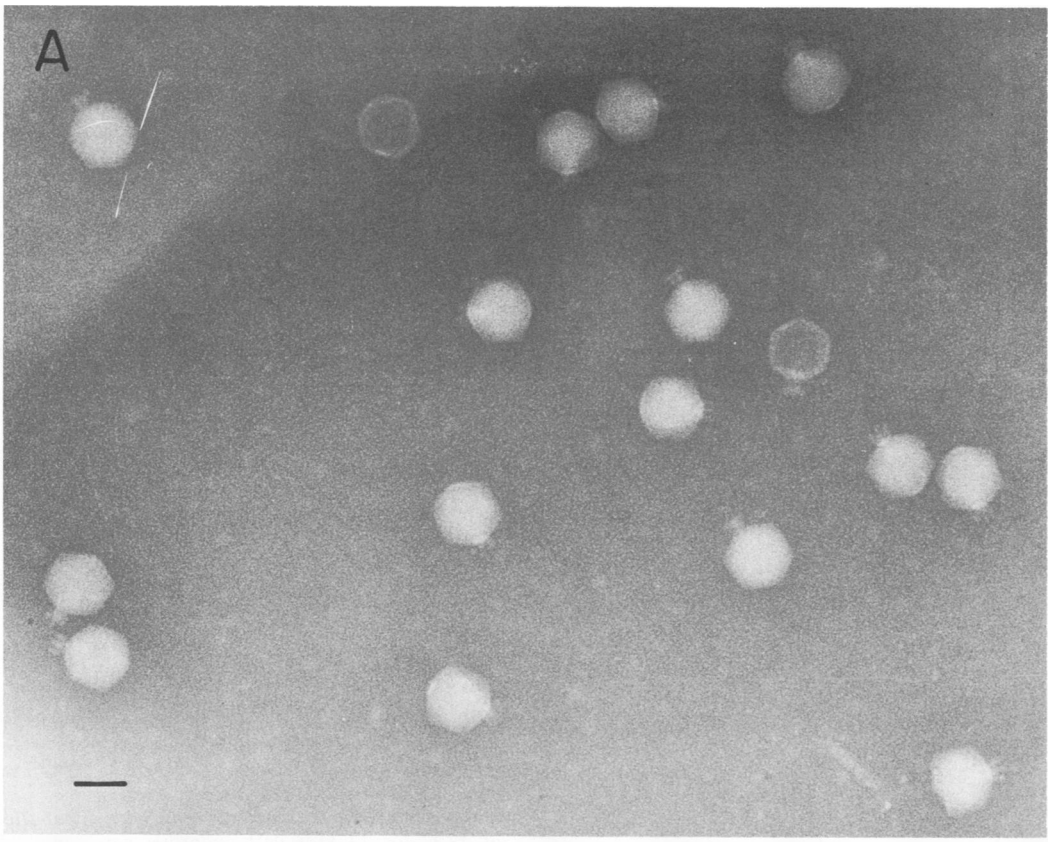



FIG. 2. *CsCl density gradient centrifugation of partially purified E. coli 263 lysate. The partially purified preparation was mixed with solid CsCl and the density was adjusted to 1.50 g/cm³ by addition of distilled water. After centrifugation at 44,000 rpm (Beckman L5-50, Ti 60 rotor) for 40 h at 20°C, the gradient was fractionated by suction through a needle puncturing the bottom of the tube, and the absorbance at 260 nm (●) and 280 nm (○) was determined.*

FIG. 3. *Electron micrograph of φ263 negatively stained with 1% phosphotungstic acid. Bar indicates 50 nm. (A) Photo micrograph of several phage particles. The particles have hexagonal symmetry and short tails composed of at least three tail spikes. Some phage particles have apparently lost their deoxyribonucleic acid so that, with the heads empty, they appear only as an outline of the complete head. (B) A 600,000-fold magnification of some φ263 phage particles. The diameter of the phage is 65 nm; the tail is 15 nm long.*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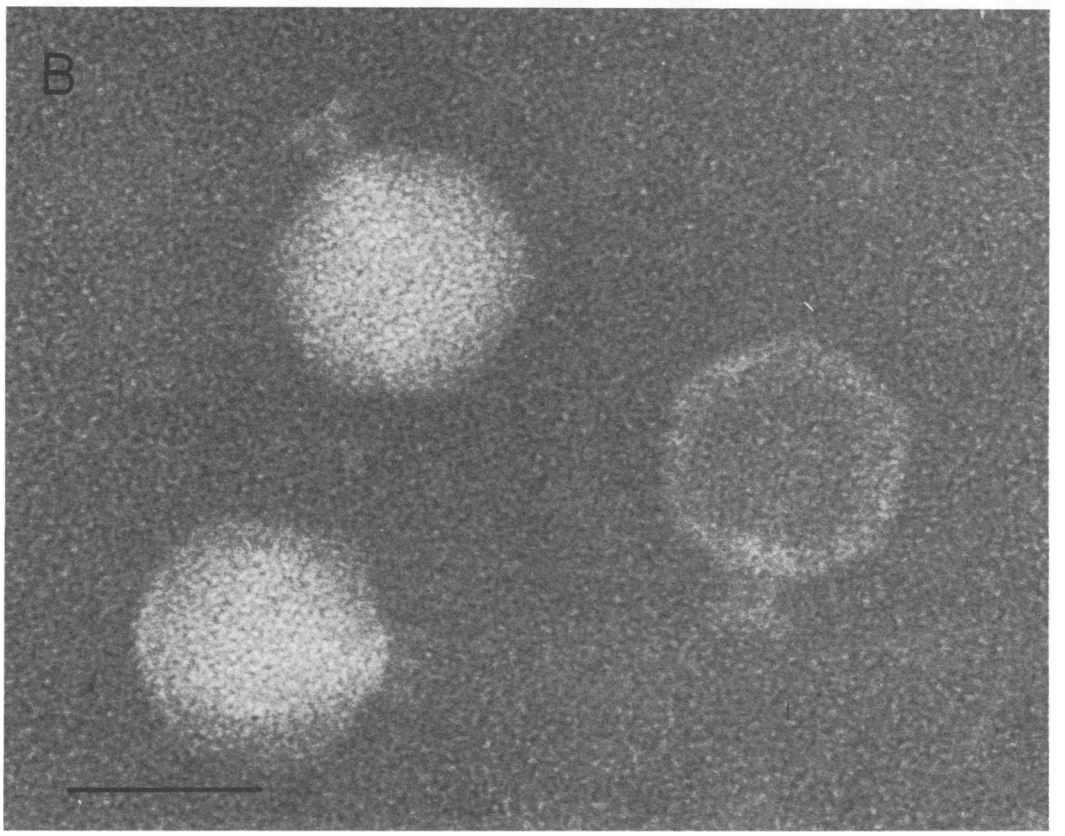
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genic. Unlike the situation with $\phi$263, screening studies with filtrates of strain B21-4 on a wide variety of enteric strains revealed a lytically sensitive host for the phage lysogenizing this strain. This phage, designated $\phi$B21-4, was recovered by plating culture filtrates of strain B21-4 on *Shigella flexneri* 2a strain M42-43. On this host in soft-agar overlayers, $\phi$B21-4 produces distinct, turbid plaques (~1 to 2 mm in diameter) typical of many temperate phages.

**Release of LT from *E. coli* 263 by thermoinduction of phage P1Cm.** Our finding that *E. coli* 263 and B21-4 carry temperate phages that can be lytically induced by mitomycin C suggested that the elevated levels of LT observed in filtrates of such induced cultures might be a reflection of a release of cell-bound toxin. To determine whether lytic induction of phage synthesis can cause such an increase in LT, *E. coli* 263 was lysogenized with the thermoinducible phage P1Cm. This phage can be specifically induced into lytic replication by elevating the incubation temperature to 42°C and inactivating the thermolabile repressor responsible for maintenance of the prophage state (2, 12). The use of mitomycin C to induce lysis of strain 263 could thus be avoided, and the levels of LT in lysates of such thermally induced P1Cm lysogens could be monitored. The results of such a thermal induction of *E. coli* 263 (P1Cm) are presented in Fig. 4. As cellular lysis resulting from lytic replication of phage P1Cm progressed, a 64-fold increase in LT activity was detected.

**LT activity of sonicates.** Because lysis resulting from bacteriophage induction results in an increase of LT in culture filtrates, it was of interest to determine the levels of preexisting LT in sterile filtrates of strains that had been disrupted by sonic oscillation. Table 2 summarizes the results of such studies of *E. coli* strains 263, B21-4, and TD286C2. When sonic oscillation of these cultures resulted in a 95% reduction of OD (about 8 min), the LT activity of filtrates had increased to levels that were typical of those of mitomycin-induced cultures. The significant drop in LT activity observed in lysates after 10 min of sonic treatment is most likely a reflection of LT inactivation caused by heat generated during sonic oscillation.

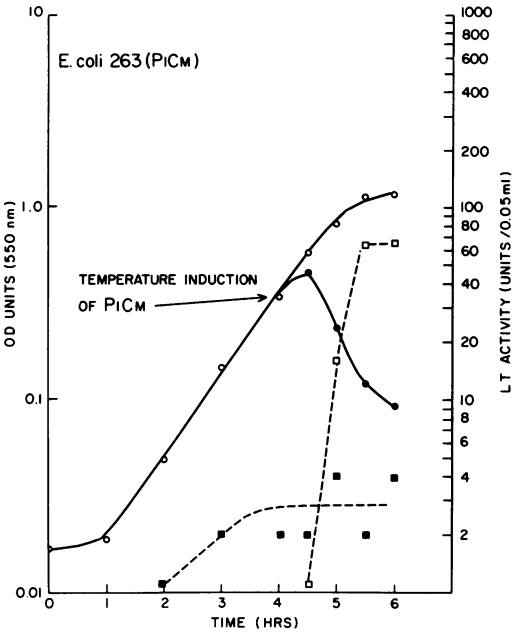


FIG. 4. *Thermal induction of E. coli 263 (P1Cm). A P1Cm lysogen of E. coli 263 was grown to a concentration of about 3 × $10^8$ cells/ml at 32°C with vigorous aeration. At this time, the culture was split, and one portion was thermally induced by incubation at 42°C for 14 min with vigorous aeration. This culture and the control portion were then incubated further at 32°C. Symbols: Control: ○, $OD_{550}$; ■, LT activity; Thermal induction: ●, $OD_{550}$; □, LT activity.*

TABLE 2. *LT activity of sonicates of E. coli*[a]

| Strain | LT activity (U/0.05 ml) after sonic oscillation for: | | | | | |
|---|---|---|---|---|---|---|
| | 0 min | 2 min | 4 min | 6 min | 8 min[b] | 10 min |
| 263 | 10 | 80 | 80 | 80 | 320 | 320 |
| | 8 | 32 | 64 | 64 | 128 | 64 |
| B21-4 | 10 | 80 | 80 | 160 | 160 | 40 |
| | 2 | 40 | 40 | 80 | 160 | 80 |
| TD286C2 | 10 | 40 | 80 | 160 | 320 | 20 |
| | 10 | 40 | 80 | 40 | 40 | 40 |

[a] Duplicate experiments were performed for each strain.

[b] $OD_{550}$ was reduced by 95% at 8 min.

## DISCUSSION

In a previous study, Isaacson and Moon (6) demonstrated that growth of the porcine enterotoxigenic *E. coli* strain 263 in the presence of mitomycin C resulted in a significant increase in LT activity of supernatant culture fluids. They proposed that addition of mitomycin C probably derepresses some regulatory toxin gene(s) on Ent, causing an induction of synthesis of LT by *E. coli* 263 and other similar strains.

The data from our studies also have shown an increase in LT activity of filtrates following mitomycin C treatment of enterotoxigenic *E. coli*. Further studies of this phenomenon in *E. coli* strains 263 and B21-4, however, revealed additional findings. Time course studies of the effect of mitomycin C (Fig. 1) provided evidence of cell

lysis concomitant with the increase in LT activity. Moreover, we subsequently were able to demonstrate that this lytic event reflected the induction of bacteriophage synthesis. Both *E. coli* 263 and B21-4 were shown to be lysogens carrying inducible phages.

On the basis of these observations, we offer an alternative mechanism for explaining the increased LT levels in supernatant culture fluids of *E. coli* strains treated with mitomycin C. We believe that mitomycin C may not specifically induce synthesis of LT; rather it induces vegetative replication of phages that may lysogenize such strains. As lysis proceeds, cellular release of preformed LT from the periplasmic space of the outer cell membrane (1) occurs, thus accounting for the increased levels of LT in filtrates. Such a mechanism is consistent with our findings as well as those previously reported by Isaacson and Moon (6). Their data from chromatographic, antitoxin, and heat lability studies indicate that LT from mitomycin C-treated cells did not differ significantly from those of LT from untreated cultures in its molecular size and other properties. Furthermore, they showed that addition of chloramphenicol to mitomycin C-treated cultures inhibits the expected increase in LT activity. Chloramphenicol, an inhibitor of protein synthesis, likewise prevents bacteriophage synthesis and subsequent cell lysis (9).

Both of these observations are consistent with our belief that preexisting toxin molecules are being released from bacterial cells via phage lysis. Additional support is provided by our experiments with the thermal induction of phage P1Cm in *E. coli* 263 (P1Cm). This strain of 263 was constructed to provide us with a system for precisely inducing a known phage into lytic growth via the specific inactivation of a thermolabile repressor. Since only the repressor specific for P1Cm phage and not the LT genes of Ent are affected by the elevation of temperature to 42°C, any rise in LT levels following phage induction should reflect the lytic release of preexisting cell-bound LT. As shown in Fig. 4, a 64-fold increase in LT activity was detected following thermal induction of phage P1Cm. Thus, the level of LT activity in such preparations approximates that achieved by mitomycin C induction of *E. coli* 263. Moreover, elevated toxin levels have also been detected in sonicates of *E. coli* 263 culture grown under similar conditions (Table 2). LT levels of filtrates resulting from some disruption of enterotoxigenic strains are comparable to levels detected after mitomycin C induction.

Early in these studies we considered the possibility that temperate phages associated with toxigenic strains of *E. coli* may have incorporated genes for enterotoxin production into their genome. Since the structural genes for LT undoubtedly represent a small portion of the Ent plasmid (17) it is likely that such LT-converting phages exist. Studies in which we have lysogenized nontoxigenic strains with various phages isolated from toxigenic *E. coli* are currently being pursued in an effort to isolate such LT-converting phages.

The use of lytic induction of bacteriophages for cellular release of toxins from bacteria may have some applications to the characterization of toxins. Preliminary studies with P1Cm lysogen of *Shigella dysenteriae* 1 strain 60R suggest that shiga toxin also can be released by induction of phage lysis (P. Gemski, unpublished data). This approach may therefore provide an alternative to alkaline extraction or mechanical disruption of cells, which are currently employed for initial enrichment and isolation of the toxin. In addition, shiga toxin isolated from phage lysates could differ from that isolated by the conventional methods and, thus, could provide a useful approach for delineating structurally the cytotoxic, neurotoxic, and enterotoxic activities of the shiga toxin complex.

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