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Bacteriophage Conversion of Heat-Labile Enterotoxin in *Escherichia coli*

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A temperate phage designated $o\beta_1$ (omicron beta) was mitomycin C induced and isolated from heat-labile enterotoxin (LT)-producing *Escherichia coli* E2631-C2. Phage $o\beta_1$ infected the nonlysogenic, nontoxigenic, mitomycin C-sensitive strain of *E. coli* K-12 (CSH38) and converted it to lysogeny and enterotoxigenicity. After the establishment of lysogeny, *E. coli* CSH38($o\beta_1$) produced LT and phage particles at maximal levels following mitomycin C induction. The LT Tox^+ character is carried by the temperate phage $o\beta_1$.

Enterotoxigenic *Escherichia coli* can produce two different kinds of enterotoxin: heat labile (LT) and heat stable (ST). It has been reported that the genes which code for the production of these toxins are carried on plasmids designated Ent (7, 19, 20, 21, 22). Isaacson and Moon (11) have reported that production of LT by enterotoxigenic *E. coli* is stimulated by the addition of mitomycin C to the culture medium. This observation has been confirmed for some, but not all, enterotoxigenic LT^+ strains of *E. coli* (16). Isaacson and Moon (11) have proposed that the addition of mitomycin C causes a derepression of the LT gene(s) on the Ent plasmid, thus causing an increase in LT synthesis. However the effect of mitomycin C on LT synthesis is not universal, and, therefore, the molecular mechanism(s) of mitomycin C stimulation of LT synthesis remains to be elucidated.

Since mitomycin C is also potent inducer of temperate bacteriophage in lysogenic bacteria (14, 17), we investigated whether enterotoxigenic *E. coli* were lysogenic and, if so, whether the induction of temperate phage was related to LT production. This communication reports the isolation of a temperate phage, $o\beta_1$ (omicron beta), from an enterotoxigenic LT^+ strain of *E. coli* that carries the genetic determinant *tox* for LT. Infection of nonlysogenic, nonenterotoxigenic, sensitive *E. coli* CSH38 with $o\beta_1$ resulted in phage conversion to enterotoxigenicity.

MATERIALS AND METHODS

Bacterial strains. Enterotoxigenic *E. coli* E2631-C2, LT producing, was provided by G. K. Morris, Center for Disease Control, Atlanta, Ga. Non-enterotoxigenic strains *E. coli* K-12 (CSH38) (Trp Thi) and CSH38 [Thi Su2($SuII^+$)], which grows on lactose but not melibiose at 42°C, were obtained from Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (15).

Media. *E. coli* strains were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) with shaking unless otherwise specified. Minimal agar plates contained: K_2HPO, 10.5 g; KH_2PO_4, 4.5 g; $(NH_4)_2SO_4$, 1 g; sodium citrate, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; thiamine hydrochloride, 0.005 g; and agar, 15 g/liter of distilled water. LB agar contained: tryptone (Difco), 10 g; yeast extract (Difco), 5 g; NaCl, 10 g; and agar, 15 g/liter of distilled water. LB soft agar contained the same components as LB agar, except that the concentration of agar was 0.8%.

Preparation of purified LT and anti-LT antiserum. LT was purified from culture filtrates of enterotoxigenic *E. coli* RIMD 0509104 (Laboratory for Culture Collection, Research Institute for Microbial Disease, Osaka University) grown in tryptic soy broth (Difco). Purification was carried out by successive column chromatography on diethylaminoethyl-cellulose, Sephadex G-200, and hydroxyapatite. A detailed description of the purification will be published elsewhere (Takeda, Taga, and Miwatani, in preparation). The final preparation of LT gave one band after polyacrylamide disc gel electrophoresis and immunoelectrophoresis against anti-LT sera. The purified LT was biologically active in the Chinese hamster ovary cell assay (10) and the S49 mouse lymphosarcoma cell assay (F. E. Ruch, J. R. Murphy, L. Graf, and M. Field, J. Infect. Dis., in press) for enterotoxin.

Antiserum against the purified LT was obtained by immunizing a rabbit several times with 50 μg of purified LT emulsified in Freund complete adjuvant. The antiserum obtained neutralized the activity of the purified LT in the Chinese hamster ovary cell assay. Only one precipitin line was observed after radial immunodiffusion of crude LT preparations against the anti-LT sera.

Counterimmunoelectrophoresis of LT. Coun-

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terimmunoelectrophoresis was carried out on a 0.8% Noble agar plate with 20 mM barbital buffer (pH 8.6). Five microliters each of antigen and anti-LT antiserum were placed in wells (2.5-mm diameter) cut in the gel plate. The distance between the two wells was 9 mm. Electrophoresis was performed at a constant current of 2 mA/cm of gel for 3 h. The precipitin lines formed were stained with Coomassie brilliant blue after the gel plate was soaked for 18 h in a 0.04% NaCl–0.4% sodium borate solution and then dried. By this method, the anti-LT antisera preparation used in this study formed a precipitin line with 1.6 μg or more of purified LT per ml.

S49 mouse lymphosarcoma cell assay of LT. Assay of LT on S49 mouse lymphosarcoma cells was carried out as described previously (Ruch et al., in press). S49 cells (3×10^6/ml) were suspended in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, Eagle minimal medium, nonessential amino acids, 2 mM glutamine, 50 IU of penicillin per ml, 50 μg of streptomycin (Flow Laboratories, Rockville, Md.), per ml, 0.01 mM Ro-20-1724 (Hoffman LaRoche, Inc., Nutley, N.J.), and 20 μg of gentamycin sulfate (Sigma Chemical Co., St. Louis, Mo.), in 200-μl portions per well in microtiter plates (TS-FB-96, Linbro Scientific, Hamden, Conn.). Culture supernatant fluids of *E. coli* were added to appropriate wells in 10- to 20-μl portions. Microtiter plates were incubated at 37°C in an atmosphere containing 7% CO_2. The presence of biologically active toxin was detected colorimetrically, i.e., by the pink color of the tissue culture medium due to inhibition of S49 cell growth. The absence of biologically active toxin was detected by the color change (pink to yellow) of the phenol red pH indicator in the culture medium after 56 to 72 h of incubation.

Phage purification. Phage were harvested and purified as previously described (24). Crude supernatant material of *E. coli* grown in the presence of 2 μg of mitomycin C per ml was adjusted to pH 8.0 and chilled to 4°C. NaCl and polyethylene glycol 6000 were added to final concentrations of 0.5 M and 5.5%, respectively. After mixing for 1 h and standing overnight at 4°C in 2-liter separatory funnels, the phage was sedimented by centrifugation at 4,5000 × *g* for 15 min in a Beckman JA-10 rotor. The phage-containing pellet was suspended in a buffer (pH 8.0) containing 10 mM tris(hydroxymethyl)aminomethane-hydrochloride, 10 mM NaCl, and 10 mM $MgCl_2$ and centrifuged for 35 min at 35,000 × *g* in a Beckman JA-20 rotor to sediment the bacterial debris. The phage-containing supernatant fluid was layered on a CsCl block gradient made up of 1.5 ml of CsCl at $\rho = 1.7$ g/cm^3, 2.0 ml of CsCl at $\rho = 1.5$ g/cm^3, and 4.0 ml of CsCl at $\rho = 1.3$ g/cm^3 and centrifuged for 90 min at 70,000 × *g* in a Beckan SW25.1 rotor at 15°C. The phage banded at the interface of CsCl $\rho = 1.3$ g/cm^3 and CsCl $\rho = 1.5$ g/cm^3 and was removed with a needle and syringe.

Electron microscopy. Purified $o\beta_1$ phage was diluted in a buffer (pH 8.0) containing 10 mM tris(hydroxymethyl)aminomethane-hydrochloride, 10 mM NaCl, and 10 mM $MgCl_2$ and dropped onto the surface of Formvar-supported, carbon-coated 200-mesh copper grids. After 30 s the excess phage suspension was drawn off from the side of the grids with bibulous paper, and, after drying, the grids were stained with 1% phosphotungstate (pH 7.0) for 15 s. Negative-stained preparations were examined in a Phillips model 300 electron microscope at an accelerating voltagle of 60kV.

Phage titration. Bacterial lysates were serially diluted in tryptic soy broth, mixed with 0.1 ml of *E. coli* CSH38 (ca. 10^7 colony-forming units), and incubated in the presence of 1 mM $CaCl_2$ at 37°C for 15 min. A 2.5-ml portion of LB soft agar was added and mixed thoroughly, and the suspension was poured onto the surface of an LB agar plate. After overnight incubation at 37°C, plaques were counted.

Titration of LT concentration. Twofold serial dilution of samples were made in 10 mM phosphate buffer (pH 7.0), and 5-μl samples were used for counterimmunoelectrophoresis. The reciprocal of the highest dilution that gave a precipitin line against anti-LT serum was determined, and this value was multiplied by 1.6 μg/ml, since 1.6 μg of LT per ml was the lowest concentration that gave a precipitin line. Standard concentrations of 0.8, 1.6, 3.2, 6.4, and 12.8 μg of LT per ml were run on each counterimmunoelectrophoresis plate.

Treatment of purified $o\beta_1$ with deoxyribonuclease. Purified $o\beta_1$ was incubated in the presence of 20 μg of bovine pancreatic deoxyribonuclease I (Calbiochem, La Jolla, Calif.) per ml, 10 mM $MgCl_2$, and 10 mM potassium phosphate buffer (pH 7.0), in a total volume of 0.2 ml at 37°C for 2 h. As a control, purified phage were incubated without deoxyribonuclease.

RESULTS

Stimulation of LT synthesis by the addition of mitomycin C to the growth medium was studied with 57 strains of enterotoxigenic LT$^+$ *E. coli*. In most of the strains tested, greater concentrations of LT were found in the culture supernatant fluids after 2 μg of mitomycin C per ml was added at an absorbance at 590 nm (A_{590}) of 0.3 compared with the amount of LT produced in the absence of the drug. Moreover, it was found that, when 2 μg of mitomycin C per ml was added at an A_{590} of 0.3, the final A_{590} reached by most of the enterotoxigenic strains tested was significantly less than that obtained in its absence. With some strains that were tested, almost no increase in the A_{590} of the culture was observed after the addition of mitomycin C. A kinetic study of cell growth revealed that the lower A_{590} obtained with some strains of enterotoxigenic *E. coli* was not due to growth inhibition by mitomycin C but, rather, to the lysis of growing cells after a latent period. The typical pattern of lysis observed with one of the LT$^+$ strains, E2631-C2, is shown in Fig. 1. After the addition of mitomycin C to 2 μg/ml at an A_{590} of 0.3, the cell density increased exponentially for approximately 1 h and then decreased. Because these data indicated that temperate phage

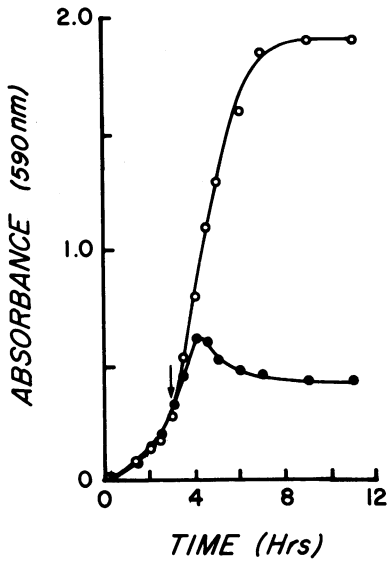

FIG. 1. *Effect of mitomycin C on the growth of E. coli E2631-C2 (LT+). Cells were grown in tryptic soy broth with shaking at 37°C. A_{590} was determined periodically in a Bosch & Lomb Spectronic 20. The arrow indicates the time of mitomycin C addition to 2 μg/ml. Symbols: ○, without mitomycin C; ●, with mitomycin C.*

were induced, culture supernatant fluids of *E. coli* E2631-C2 grown in the presence of 2 μg of mitomycin C per ml were prepared by centrifugation and sterilized with chloroform. A 5-μl portion was then spotted on each of 45 different non-enterotoxigenic *E. coli* lawns growing on LB agar. After overnight incubation at 37°C, the largest plaques were found on lawns of *E. coli* CSH31 and CSH38.

To test the possibility that temperate phage released after mitomycin C induction were related to the enterotoxigenicity of *E. coli* E2631-C2 (LT+), the sensitive strain *E. coli* CSH38 (LT−) was incubated in sterile supernatant fluids of E2631-C2 (LT+). $CaCl_2$ was added to 1 mM to facilitate phage attachment. After 15 min of incubation at 37°C, 5 ml of fresh tryptic soy broth was added, and incubation with shaking was continued for 6 h. The culture was then streaked on 0.2% melibiose minimal agar plates for single colonies. Since *E. coli* CSH38 (LT−) does not grow at 42°C on melibiose, the plates were incubated at this temperature for 24 h before incubation at 37°C to insure that the colonies that grew at 37°C were of the CSH38 phenotype. One hundred one colonies were randomly picked and grown separately in tryptic soy broth at 37°C with shaking. Mitomycin C (2 μg/ml) was added to each culture at an A_{590} of 0.3, and incubation was continued for 15 h. Culture supernatant fluids were prepared by centrifugation and tested for their reactivity against anti-LT sera by counterimmunoelectrophoresis. Two cultures were found to be sensitive to mitomycin C-induced lysis, and their cell-free culture supernatant fluids were precipitin positive. These cultures were designated TM38-1 and TM38-2. Crude cell-free lysates of the parental *E. coli* CSH38 (LT−) grown in the presence and absence of 2 μg of mitomycin C per ml were not precipitin positive after counterimmunoelectrophoresis. In addition, the culture supernatant fluids of mitomycin C-induced TM38-1 and TM38-2 were found to be positive in the S49 mouse lymphosarcoma cell assay for enterotoxin.

These results strongly indicated phage conversion of *E. coli* CSH38 (LT−) to CSH38 (LT+). The enterotoxigenic TM38-1 was grown in 4 liters of tryptic soy broth in a Microferm fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) at 37°C, sparged with air at 3 liters/min, and stirred at 500 rpm. Mitomycin C was added to 2 μg/ml at an A_{590} of 0.3, and after 16 h of incubation phage were purified from the culture supernatant fluid as described in Materials and Methods. Negative-stained preparations of purified phage examined in an electron microscope revealed a small phage with a 40-nm-diameter head and a noncontractile tail approximately 130 nm long. We named this phage $o\beta_1$ (omicron beta).

TM38-1, hereafter called *E. coli* CSH38($o\beta_1$), was lysogenic and sensitive to mitomycin C induction, whereas the parent CSH38 is not lysogenic (Fig. 2). Phage $o\beta_1$ was induced by the addition of mitomycin C to 2 μg/ml at an A_{290} of 0.3, and lysis occurred approximately 3 h later. The lysis of CSH38 ($o\beta_1$) corresponded to the release of phage (Fig. 3) and the accumulation of LT in the culture medium (Fig. 4). Without the addition of mitomycin C, phage were released spontaneously from CSH38($o\beta_1$). This spontaneous release was also related to the accumulation of LT in the culture medium (Fig. 3 and 4).

CSH38($o\beta_1$) was mitomycin C induced, and phage $o\beta_1$ was purified through CsCl centrifugation as previously described. Approximately 6×10^{10} plaque-forming units of phage $o\beta_1$ was incubated with 1×10^{11} colony-forming units of CSH38 in 0.2 ml of tryptic soy broth, and incubation was continued for 5 h with shaking. The culture was then streaked on 0.2% melibiose minimal plates for single colonies. The plates were incubated at 42°C for 24 h and then at 37°C for 24 h as previously described, and then 50 colonies were randomly picked, grown separately in tryptic soy broth, and induced with

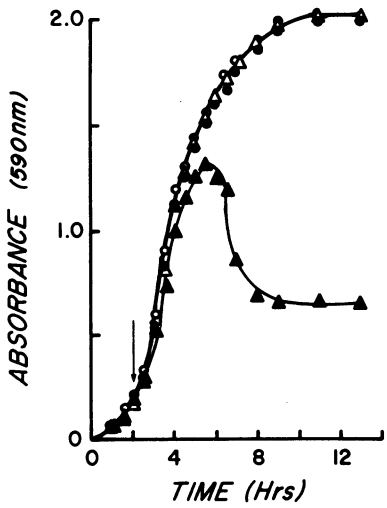

FIG. 2. *Effect of mitomycin C on the growth of E. coli CSH38 and CSH(oβ1). Cells were grown in tryptic soy broth with shaking at 37°C. A_{590} was determined periodically. The arrow indicates the time of mitomycin C addition to 2 μg/ml. Symbols: ○, CSH38 without mitomycin C; ●, CSH38 with mitomycin C; △, CSH38(oβ1) without mitomycin C; ▲, CSH38(oβ1) with mitomycin C.*

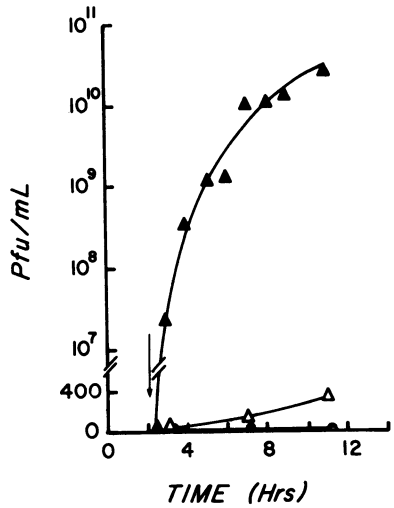

FIG. 3. *Titration of phage released from E. coli CSH38 and CSH38(oβ1) grown in the presence and absence of 2 μg of mitomycin C per ml. The arrow indicates the time of mitomycin C addition. For an explanation of symbols, see the legend to Fig. 2.*

mitomycin C; after centrifugation, supernatant fluids were tested for LT by counterimmunoelectrophoresis. All 50 colonies tested were found to be LT$^+$. To eliminate the possibility that transformation by copurified plasmids was related to the conversion of CSH38 (LT$^-$) to

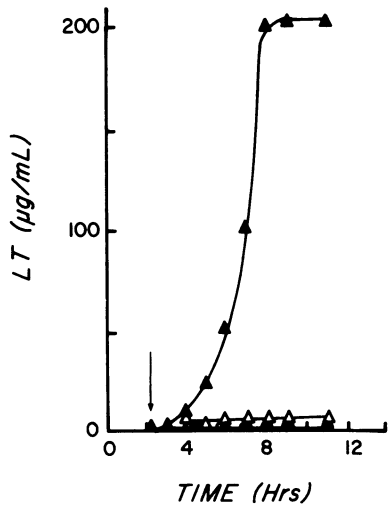

FIG. 4. *Production of LT enterotoxin by E. coli CSH38 and CSH38(oβ1) grown in the presence and absence of 2 μg of mitomycin C per ml. The arrow indicates the time of mitomycin C addition. For an explanation of the symbols, see the legend to Fig. 2.*

CSH38 (LT$^+$), CsCl-purified phage $o\beta_1$ was treated with deoxyribonuclease and then incubated with CSH38 (LT$^-$) as described above. This treatment did not affect the rate of conversion, and all 50 colonies tested were found to be LT$^+$.

DISCUSSION

The addition of low concentrations of mitomycin C to some enterotoxigenic LT-producing strains of *E. coli* has resulted in the stimulation of LT production (11, 16). The precise mechanism(s) of this stimulation, however, remains unknown. Issacson and Moon (11) have suggested that mitomycin C induces preferrential synthesis of a plasmid-carried LT gene(s). This is an attractive hypothesis, since it has been known for several years that the gene(s) which codes for LT synthesis is carried on the Ent plasmid (7, 19–22) and that mitomycin C has been shown to stimulate the synthesis of colicin E1 (4). It is clear that mitomycin C has many effects, since low concentrations are also known to induce the vegetative growth of temperate phage in lysogenic bacteria (14, 17) and to stimulate the production of ColE1 plasmids in *Proteus mirabilis* (4).

We have confirmed the effect of mitomycin C stimulation of LT production by some enterotoxigenic strains of *E. coli* and have shown that, in the case of *E. coli* E2631-C2 (LT$^+$), mitomycin C treatment also induces vegetative growth of a prophage. We have named the temperate phage isolated from strain E2631-C2

"$o\beta_1$." The sensitive strain *E. coli* CSH38 can be converted to toxigenicity after infection with the temperate phage $o\beta_1$. The production of LT by the lysogen CSH38 ($o\beta_1$) has been detected by counterimmunoelectrophoresis against anti-LT sera and by the toxicity of culture supernatant fluids in the S49 mouse lymphosarcoma cell assay for enterotoxin (Ruch et al., in press). As in the case of E2631-C2 (LT^+), the maximal production of LT by CSH38 ($o\beta_1$) occurred after mitomycin C induction. The appearance of LT correlated with the lysis of CSH38 ($o\beta_1$) and the appearance of phage $o\beta_1$ particles in the culture medium.

It is widely known that the synthesis of both LT and heat-stable enterotoxin by enterotoxigenic *E. coli* is, in many cases, controlled by the Ent plasmids (7, 19–23). The molecular size of the Ent plasmids has been studied, and several heterologous plasmids have been reported (7). The recognition of the size heterogenicity among the Ent plasmids in *E. coli* has led to the speculation that the genetic information for LT production might be located on a translocatable element. To date there is no direct evidence that this is the case. However, in the case of R-factors, a tetracycline-resistant element (Tn*10*) has been shown to translocate from a plasmid to the genome of *Salmonella* phage P22 (12). In addition, the translocation of R-determinants from R-plasmids to other plasmids, bacteriophage genomes, or the bacterial chromosome has been observed for kanamycin (Tn*5*), chloramphenicol (Tn*9*), ampicillin (Tn*1*, Tn*2*, Tn*3*), and ampicillin-sulfonamide-streptomycin (Tn*4*) (1, 3, 5, 6, 8, 9, 13, 18). The possible translocation of the LT *tox* determinant from $o\beta_1$ is currently under investigation.

Since not all LT-producing strains of enterotoxigenic *E. coli* are sensitive to mitomycin C stimulation of LT production, it is possible that the *tox* determinant is carried on a prophage genome in some of the strains that are sensitive to mitomycin C stimulation and on an Ent plasmid in other strains. This hypothesis is also under current investigation.

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ADDENDUM IN PROOF

We have recently observed that LT toxin produced by *E. coli* CSH38($o\beta_1$) is not active in the Y-1 adrenal cell assay for enterotoxin, but is active in the Chinese hamster ovary and S49 cell assays for enterotoxin. This anomaly is under investigation.

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