INFECTION AND IMMUNITY, Oct. 1993, p. 4510–4513
0019-9567/93/104510-04$02.00/0

Vol. 61, No. 10

Mitomycin-Induced Synthesis of a Shiga-Like Toxin from Enteropathogenic *Escherichia coli* H.I.8

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Received 20 April 1993/Returned for modification 3 June 1993/Accepted 3 August 1993

***Escherichia coli* H.I.8, an O128 infant diarrhea isolate, produces low titers of a unique Shiga-like toxin (SLT), called SLT-IIva, which is a variant of SLT-II. We investigated induction of toxin synthesis and the putative association of a bacteriophage with toxin synthesis. Induction of broth cultures of strain H.I.8 with mitomycin yielded a 3,000-fold increase in SLT-IIva, production of a colicin, and appearance of a bacteriophage. Southern hybridization demonstrated that the genes for SLT-IIva were not carried by the bacteriophage.**

The genes for Shiga-like toxin I (SLT-I) and SLT-II are carried by bacteriophages in *Escherichia coli* strains implicated in hemorrhagic colitis and diarrhea in humans (16, 21). The genes for SLT-IIe (previously called SLT-IIv), associated with edema disease of pigs, appear not to be phage mediated (12). There is no information on association with phages of the genes for SLT-IIva, produced by *E. coli* H.I.8 (O128:B12), recovered from a child with diarrhea (9). SLT-IIva is highly related to SLT-II but more closely related to SLT-IIe (3). In this study we determined whether a bacteriophage released from strain H.I.8 by exposure to mitomycin carried the genes for SLT-IIva and could account for an observed increase in toxin synthesis.

SLT-producing strains of *E. coli* used in this study are listed in Table 1. The *E. coli* K-12 strain C600 was a SLT-negative control strain; the *E. coli* B and *E. coli* K-12 strains C600 and 711, respectively, were used as host strains for bacteriophage propagation. The *E. coli* B strain was also used as an indicator strain in colicin assays. *E. coli* DH5α(pVGT15) contains plasmid pUC19 with a 2.0-kb *Ava*II-*Hin*cII insert of the *slt*-IIva genes and was kindly donated by V. P. J. Gannon, Animal Diseases Research Institute, Agriculture Canada, Lethbridge, Alberta, Canada.

E. coli strains were grown aerobically at 37°C on blood agar plates or in brain heart infusion broth (BHIB) (Difco, Detroit, Michigan) and, for induction studies, in syncase broth (14) or BHIB with shaking. For bacteriophage propagation, overlays were made on Luria-Bertani (20) or MacConkey agar.

SLT was quantified by a Vero cell assay, and toxin was neutralized as described by MacLeod and Gyles (11). Polyclonal antiserum was prepared in two 3-month-old New Zealand White rabbits immunized as described by Ritchie (19) with a polymyxin B extract of *E. coli* DH5α(pVGT15) (11).

For optimization of induction, 25-ml volumes of syncase broth were inoculated with 1 ml of an overnight broth culture of strain H.I.8. The flasks were incubated at 37°C with shaking, and the optical density at 600 nm was measured hourly. Mitomycin was added to final concentrations of 0.1, 1.0, and 2.0 μg ml^{-1} during mid-logarithmic-phase growth. Cell lysis was monitored spectrophotometrically, and the toxin content of culture supernatants and sonicated cell pellets was measured by the Vero cell assay. The mitomycin concentration which resulted in the greatest amount of cell lysis during a 6-h incubation period was selected as optimal. By similar procedures, the optimum time for addition of mitomycin was determined by addition to broth cultures of mitomycin at a final concentration of 2.0 μg ml^{-1} at 2, 3, and 4 h postinoculation.

For induction of large quantities of culture, mitomycin was added to mid-logarithmic-phase cultures of strain H.I.8 in BHIB to a final concentration of 2.0 μg ml^{-1}. Following maximal lysis, the remaining intact bacteria were lysed by addition of chloroform (1%, vol/vol) and the cellular debris was removed by centrifugation (11,000 × *g* for 20 min at 4°C). The supernatant was stored at 4°C over chloroform.

Phage particles in the culture supernatant (25 μl) of induced cultures of strain H.I.8 were negatively stained with 2% (wt/vol) phosphotungstic acid and examined by electron microscopy. Propagation of the phage was attempted by an overlay technique (16) and by spot inoculation (22) using the indicator *E. coli* B strains 711 and C600 and a spontaneous colicin-resistant mutant of C600. Colicin yields in culture supernatants were assayed by the diffusion zone method of Mayr-Harting et al. (13).

DNA was extracted from bacteriophage particles recovered from strain H.I.8 (20). DNA from plasmid pUC18 was added as a control to the phage suspension prior to DNase treatment to monitor elimination of contaminating chromosomal DNA.

Chromosomal DNA for the polymerase chain reaction (PCR) amplification procedure was obtained from the SLT-producing *E. coli* strains (Table 1) with a DNA extractor (Applied Biosystems, Mississauga, Ontario, Canada). Chromosomal and plasmid DNAs from strain H.I.8 were extracted according to the protocols of Sambrook et al. (20) for genomic DNA extraction and plasmid DNA extraction by alkaline lysis.

For PCR amplification of SLT sequences in strain H.I.8, we used pairs of oligonucleotide primers that targeted nucleotide sequences in SLT-I, SLT-II, and SLT-IIe (7, 18). For PCR amplification of SLT-IIva gene sequences, we used

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TABLE 1. Characterized SLT-producing *E. coli* strains used in the PCR

Strain	Serotype	Toxin	Reference
H30	O26:H11	SLT-I	17a
933W	O157:H7	SLT-II	22a
E32511[a]	O157:H$^-$	VT2 (SLT-II, SLTIIc)	5
412	O139:K82	SLT-IIe	4a
H.I.8	O128:B12	SLT-IIva	3

[a] Strain E32511 has now been shown to have two operons, one for SLT-II and one for SLT-IIc (20a).

primers which amplified a 184-bp product encoding the 3′ terminus of the A subunit and part of the intergenic region between the A and B subunits. The nucleotide sequences of primers were 5′-CCC CTG TTA TTG GTT TGG TTC-3′ and 5′-TAA TTG CCA CTC AAC CGG AAG-3′. For amplification of *slt*-IIva genes, the reaction mixture contained 50 mM potassium chloride, 10 mM Tris chloride (pH 8.3), 0.75 mM magnesium chloride, 200 μM each of dATP, dCTP, dGTP, and dTTP, 0.625 U of DNA polymerase (AmpliTaq; Perkin Elmer Cetus), and each primer at 0.10 μM. Samples were overlaid with 30 μl of mineral oil, heated at 72°C for 2 min, and subjected to 35 cycles of amplification. Parameters for amplification were denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 30 s. After the last cycle, the tubes were incubated at 72°C for 5 min (18).

Southern transfer and hybridization of restricted DNA fragments were as described by Sambrook et al. (20). A [α-^{32}P]dATP-labelled 0.5-kb *Hin*cII-*Pst*I fragment of pVGT15 internal to the *slt*-IIva genes was used as a probe, and prehybridization and hybridization were conducted at 42°C.

Maximum cell lysis occurred when mitomycin was added at 2.0 μg ml^{-1} during the mid-logarithmic growth phase. Yields of SLT showed a 3,000-fold increase in the supernatant and a 25-fold increase in the cell lysate of the induced culture compared with the uninduced culture. These results were reproducible in 10 replications of the experiment.

The toxin released by mitomycin-induced cultures of strain H.I.8 was shown to be SLT-IIva by neutralization of the Vero cell cytotoxicity with the polyclonal anti-SLT-IIva rabbit antiserum. Ten cytotoxic doses of toxin in culture supernatants were neutralized at a serum dilution of 1/78,125 by each of two serum samples. Neutralization of the Vero cell toxicity associated with strain H.I.8 by antitoxin specific for SLT-IIva showed that SLT-IIva was the only SLT produced. Interestingly, previous studies had suggested that SLT-IIva was less immunogenic than other SLTs (2, 8), but the high titer of neutralizing antiserum obtained in this study suggests that previous results were the product of low toxin production by strain H.I.8 compared with the recombinant strain DH5α(pVGT15) (3).

When strain H.I.8 was screened for *slt* sequences by PCR amplification, the 184-bp product specific for SLT-IIva was detected and no amplification product was obtained with primers specific for the other *slt* genes. These results indicate that *slt*-IIva was the only *slt* operon present in strain H.I.8. PCR amplification showed that other *slt* operons were absent from strain H.I.8, and Southern hybridization of *Hin*dIII digests of chromosomal DNA demonstrated that there was only one *slt*-IIva operon. A single 4.6-kb *Hin*dIII fragment hybridized with the SLT-IIva probe, a finding that was consistent with the restriction map of *slt*-IIva (3). We

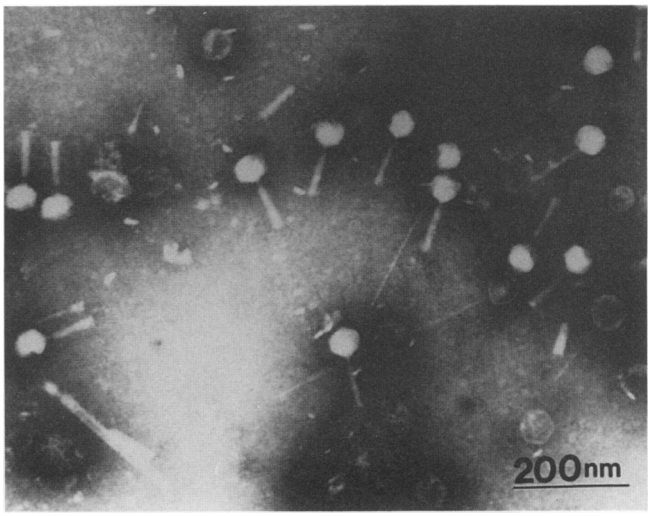

FIG. 1. Electron micrograph of a bacteriophage isolated from mitomycin-induced cultures of strain H.I.8 and purified by glycerol gradient ultracentrifugation. The phage suspension was negatively stained with 2% (wt/vol) phosphotungstic acid on formvar-coated copper grids.

confirmed by Southern hybridization that the *slt*-IIva genes were located on the chromosome and not on a plasmid.

Electron microscopic examination of supernatants from mitomycin-induced cultures of strain H.I.8 showed phage particles of one morphologic type, with icosahedral heads approximately 50 nm in diameter and contractile tails approximately 90 nm in length (Fig. 1). The phage was designated ϕ128 and was present at 10^8 particles per ml of culture supernatant.

Attempts to plaque the phage on indicator strains were unsuccessful. A colicin-resistant mutant of C600 was included among the indicator strains because the formation of plaques may have been hindered by colicin production by strain H.I.8. However, no plaques were observed with this host strain.

The DNA from phage particles recovered from supernatants of mitomycin-induced cultures of H.I.8 appeared to be of a single homogeneous type, as observed by agarose gel electrophoresis. The size of the phage genome was approximately 60 kb. When phage DNA was digested with *Hin*dIII (Fig. 2A) and hybridized with the *slt*-IIva probe, no signal was evident in the digest of the phage DNA (Fig. 2B, lane 4). The faint signal associated with the uncut phage DNA (Fig. 2B, lane 3) may represent contaminating chromosomal DNA which was incompletely removed by DNase treatment. Evidently, the highly sensitive probe detection system identified a small amount of residual chromosomal DNA. The substantial reduction in signal associated with DNase-treated phage DNA (Fig. 2B, lane 3 [cf. lane 1]) indicates that the signal was derived from DNA that was not packaged in the phage particle.

Strain H.I.8 possessed two large plasmids, and plasmid DNA was tested with the *slt*-IIva probe (photograph not shown). No hybridization was detected.

Previous attempts to induce phages from strain H.I.8 were unsuccessful (3), but in the present study, phage ϕ128 was consistently induced upon exposure to mitomycin. The 3,000-fold increase in SLT-IIva is not consistent with increased gene dosage due to induction of a temperate bacte-

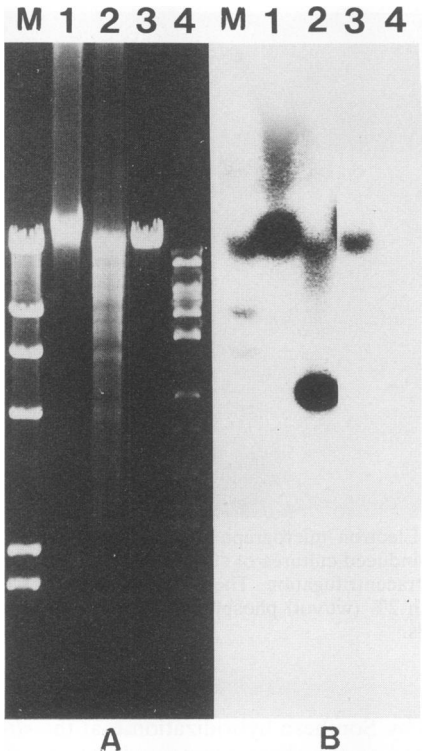

FIG. 2. (A) Hybridization of a 0.5-kb *slt*-IIva probe to chromosomal DNA fragments and phage DNA (prepared with DNase treatment) fragments separated on a 0.8% agarose gel. (B) Autoradiograph after hybridization. The gel was run initially at 90 V for 10 min and then at 70 V (0.030 A) for 3 h. Lanes: M, *Hin*dIII-digested bacteriophage lambda DNA (BRL); 1, undigested chromosomal DNA; 2, *Hin*dIII digest of chromosomal DNA; 3, undigested phage ϕ128 DNA prepared with DNase treatment; 4, *Hin*dIII-digested phage ϕ128 DNA prepared with DNase treatment.

riophage. At best, this phenomenon could explain only a small percentage of the increase. When toxin genes were carried by lysogenic bacteriophage, induction of phage resulted in increased copy number of the toxin genes and an increase in toxin synthesis of no more than 100- to 200-fold (5). Mitomycin-induced toxin synthesis has been utilized in a colony blot assay for SLT-I- and SLT-II-producing *E. coli* (6) and for increasing toxin yield for purification of SLTs (5). Induction of toxin synthesis without induction of phages has been reported for *Shigella* strains (24) and for enterotoxigenic *E. coli* (1). Even where there is simultaneous induction of phage and increased toxin levels (4, 17, 23), toxin production may not be phage mediated. For example, Gemski et al. (4) showed that an apparent increase in synthesis of *E. coli* heat-labile enterotoxin was due to an increase in the release of cell-associated toxin into the supernatant following lysis by phage.

The observed increase in SLT-IIva could be explained by derepression of toxin synthesis. Mitomycin damages DNA by cross-linking complementary strands (10) and is likely to activate the SOS system, resulting in release of operons from LexA-mediated repression (25). If regulatory regions for *slt*-IIva contain a LexA repressor binding site, then induction of increased toxin synthesis could be explained in this manner. The low levels of toxin normally produced by this strain, compared with that produced by other strains, is consistent with repression of synthesis. O'Brien et al. (15) had suggested that gene repression might explain their observation that SLT-producing *E. coli* could be categorized into trace, low to moderate, and high producers. An alternative hypothesis which cannot be ruled out is that phage ϕ128 possesses genes for a positive activator of the genes for toxin synthesis. A less likely alternative is that mitomycin directly damages the DNA sequence for a repressor of *slt*-IIva; the reproducibility of the induction phenomenon would argue against this explanation.

We gratefully acknowledge advice from D. E. Bradley, Memorial University, St. John's, Newfoundland, Canada, on the bacteriophage of strain H.I.8. We acknowledge V. P. J. Gannon's gift of the recombinant strain containing the cloned *slt*-IIva genes and Paul Huber for technical assistance in electron microscopy and photography.

This project was funded by the Natural Sciences and Engineering Research Council and the Ontario Ministry of Agriculture and Food.

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