INFECTION AND IMMUNITY, June 1979, p. 793-797
0019-9567/79/06-0793/05$02.00/0

Vol. 24, No. 3

Heat-Labile Enterotoxin Production in Isolates from a Shipboard Outbreak of Human Diarrheal Illness

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Received for publication 26 March 1979

As reported elsewhere, an enterotoxigenic strain of *Escherichia coli* serotype O25:K98:NM was epidemiologically incriminated as the etiological agent in a shipboard outbreak of diarrheal illness. This enterotoxigenic *E. coli* strain and possibly other enteric isolates were found to produce heat-labile toxin and not heat-stable toxin. Since previous genetic analyses of enterotoxigenic *E. coli* strains producing heat-labile and heat-stable toxins have shown a plasmid location for both toxin determinants and since in this outbreak more than one bacterial strain appeared to produce only heat-labile toxin, the possibility of an extrachromosomal heat-labile toxin determinant was investigated. Results of endonuclease cleavage and hybridization experiments, as well as apparent heat-labile toxin phenotypic instability, strongly suggest a plasmid mediation of toxin production. Additionally, the stability of this heat-labile toxin production was evaluated after several traditional methods of bacterial cell preservation.

Enterotoxigenic *Escherichia coli* strains pathogenic for humans and animals were initially defined by their ability to produce a heat-stable (ST) and a heat-labile (LT) enterotoxin or to produce only ST (9, 17, 25). Toxinogenesis, as well as other potentially virulent properties, such as colonization factors and hemolysin production, has been shown to be plasmid mediated in both the strains producing only ST and the LT-ST strains (8, 9, 20, 21). Nucleic acid homology studies of a variety of these two phenotypes have shown apparently distinct genotypes as well. The data indicate that there is a higher degree of homology among plasmids mediating LT-ST production in human and porcine strains than there is among plasmids from the strains producing only ST (22). Human isolates of *E. coli* producing only LT have been from sporadic episodes of diarrhea, have been inconclusively termed pathogenic and, to date, have not been genetically defined (10, 11, 13, 16).

The report of a recent shipboard outbreak epidemiologically indicated that an *E. coli* strain producing only LT (serotype O25:K98:NM) was the pathogenic organism causing human diarrheal illness (2). Another strain isolated in Nepal and antigenically similar to this organism has recently been reevaluated as one of the newly defined enterotoxigenic serotypes (14). Three cases of illness documented in a study of travelers' diarrhea in Mexico have been associated not only with the same serotype but also with the production of LT only (13).

The transmissibility and stability of enterotoxigenicity in these strains associated with this shipboard outbreak were investigated. Based on these data and a detailed plasmid analysis, the probable location of a genetic determinant for LT production is discussed.

MATERIALS AND METHODS

Bacteriology. All bacterial strains from the outbreak were subcultured from MacConkey primary isolation medium onto heart infusion agar slants and held at room temperature in the dark after an overnight incubation at 37°C. At 2 weeks after collection, seven isolates from each MacConkey plate were tested for LT production by using Y-1 adrenal cells (18), for ST production in suckling mice (5), and for invasiveness in guinea pig eyes (19). Positive and negative control strains of *E. coli* were included in each test group. A dose response in the Y-1 adrenal cell assay to LT from this strain producing only LT was compared with the response to LT in a control strain producing LT and ST (strain H10407, serotype O78:H11), which had been isolated from cholera-like diarrheal illness in Dacca, Bangladesh (8). Each was also tested for susceptibility to neutralization by the Swiss Serum Vaccine Institute cholera antitoxin. Isolates were biochemically identified by standard methods and were serotyped by using 138 *E. coli* O antisera in 21 pools (6). Identification of the K antigen in one of the epidemic strains was performed by Betty Davis, Bureau of Laboratories, Center for Disease Control. Antibiotic susceptibilities were determined by the Kirby-Bauer method (1).

Storage. At 3 weeks after collection, a representative subsample of isolates was stocked on blood agar

base slants with paraffin corks, lyophilized and frozen at −70°C in Trypticase soy broth with 20% glycerol. Retesting at 3 to 12 months was performed on 30 epidemic strain isolates and 11 non-epidemic strain isolates stored in three ways: (i) on blood agar base slants at room temperature in the dark, (ii) lyophilized and kept at room temperature in the dark, and (iii) frozen at −70°C.

Transfer experiments. Details of conjugation procedures and selective medium preparation were described previously (24). The donor epidemic strains producing only LT were resistant to sulfathiazole (SU) and tetracycline (TC) and sensitive to nalidixic acid. The first two antibiotics were incorporated into the MacConkey agar at concentrations of 25 and 8 μg/ml, respectively, to counterselect transconjugants resistant to them, and 20 μg of nalidixic acid (the selecting antibiotic) per ml was incorporated to select for the chromosomally mediated resistance of the recipients to this drug.

Four donor strains were mated with three genotypically unique lactose-negative *E. coli* K-12 recipients which were resistant to high levels of nalidixic acid. A total of 390 resistant (SU, TC, and nalidixic acid) K-12 transconjugants were assayed for LT production. In addition, 100 presumptive transconjugants (recipients incubated with donors) were isolated without counterselecting antibiotics (SU and TC) and assayed for LT.

Plasmid analysis. The plasmid content of O25:NM isolates and K-12 transconjugants was determined by agarose gel electrophoresis of partially purified lysates (25) of 30-ml bacterial cultures. Gels were prepared and run as described by Meyers et al (12). Approximate plasmid molecular weights were determined by comparison of the distance migrated in the gel with the migration of plasmids of known molecular weight.

Purified plasmid deoxyribonucleic acid (DNA) was isolated from toxigenic and nontoxigenic O25:NM strains by centrifugation of cleared lysates in CsCl-ethidium bromide gradients. Cleared lysates were prepared as described by Elwell et al. (7). Gradients were centrifuged at 35,000 rpm and 15°C for 40 to 48 h. For digestion with *Bam*HI (Miles Laboratories) restriction endonuclease, approximately 0.5 μg of purified plasmid DNA was added to a reaction mixture containing 0.01 M $MgCl_2$, 0.1 M tris(hydroxymethyl)aminomethane (pH 7.4), and 1 μl of enzyme. The reaction was carried out at 37°C for 1 h.

RESULTS

Bacteriology. The shipboard outbreak strains were found to be *E. coli* O25:K98:NM which produced only LT and were resistant to SU and TC (2). Non-enterotoxigenic *E. coli* O25:NM resistant to SU and TC were isolated from three persons, two of whom were simultaneously harboring the enterotoxigenic strain of the same serotype and antibiotic susceptibility. Additionally, 11 organisms isolated from patients in the same outbreak, including one *Citrobacter freundii*, two *Klebsiella pneumoniae*, and eight *E. coli* strains other than the epidemic serotype, were found to produce a positive Y-1 response on initial testing. They were negative on subsequent testing, however, with one exception. All of these organisms were sensitive to SU and TC, in contrast to the epidemic strain, which was resistant. Three persons were found to be harboring one of these organisms simultaneously with the epidemic strain, and this was the only potentially pathogenic isolate from eight clinically ill individuals.

After 3 to 12 months of storage by lyophilization, freezing, and blood agar base stock slants, replica copies of 30 selected epidemic strain isolates and the 11 non-epidemic strain isolates which had given a positive Y-1 response were retested. All of the copies of the 30 epidemic strains stored by the three methods were still toxigenic on repeat testing, but only the lyophilized copy of 1 of the 11 similarly stored non-epidemic strains was still positive. When the tests were repeated, however, the single positive non-epidemic strain isolate had also become negative, so normal confirmatory procedures (e.g., neutralization) could not be performed on any of the non-epidemic strain isolates. Ten control *E. coli* cultures (five toxigenic and five nontoxigenic) were included in each assay and were reproducibly positive and negative.

A comparison of the quantity and quality of LT produced by the epidemic strain with the quantity and quality of LT produced by control strain H10407 (serotype O78:H11; LT-ST producer) established no significant differences in heat susceptibility (inactivated at 100°C for 30 min) or susceptibility to neutralization by cholera antitoxin (neutralized to higher titer). Under routine laboratory conditions of filtrate preparation, the epidemic strain produced slightly less toxin than H10407, as measured by serial dilution in the Y-1 assay. The geometric mean average of the reciprocal of the highest dilution giving a positive Y-1 assay was 16 for the epidemic strain and 32 for the control strain.

Transfer experiments. Resistances to SU and TC were transferred simultaneously from the four isolates of the epidemic strain into the various K-12 recipients. The average frequency of transfer was approximately 5.8×10^{-4}. No LT production was detected in the 390 resistant or 100 presumptive transconjugants assayed in Y-1 adrenal cells.

Plasmid analysis. The plasmid content of toxigenic and nontoxigenic O25:NM isolates was compared by agarose gel electrophoresis of purified plasmid DNA. Both strains had five plasmid bands (Fig. 1), corresponding to molecular weights of 57×10^6, 27×10^6, 4.2×10^6, 2.1×10^6, and 1.5×10^6. No plasmid unique to the toxigenic strain was apparent. Electrophoresis of partially

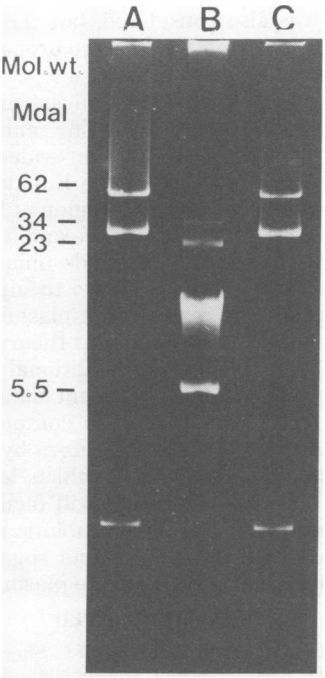

FIG. 1. *Agarose gel electrophoresis of toxigenic and nontoxigenic E. coli O25:K98:NM isolates. Lane A, Toxigenic isolate E2539C1. Lane B, Molecular weight standards: plasmid R1, 62 × 10⁶; RP4, 34 × 10⁶; Sa, 23 × 10⁶; RSF1010, 5.5 × 10⁶. Lane C, Nontoxigenic isolate E2539C3. Mol wt, molecular weight; Mdal, megadalton.*

purified lysates of Tc^r Sm^r *E. coli* K-12 transconjugants contained the 57-megadalton plasmid (Fig. 2), whereas some also contained 2.1- and 4.2-megadalton plasmid bands (possibly monomeric and dimeric forms of the same plasmid). No phenotype could be assigned to the smaller plasmid species.

As a further comparison of the plasmid content of the LT-positive and LT-negative variants, purified plasmid DNA from each strain was digested with the restriction endonuclease from *Bacillus amyloliquefaciens* H (*Bam*HI), and fragments were electrophoresed on an agarose gel. Identical banding patterns should have been observed if the plasmids in the two strains are the same. Figure 3 shows, however, that the banding pattern of the LT-positive strain (Fig. 3, lane B) is more complex than that of the LT-negative strain (Fig. 3, lane A). This is consistent with the presence of two plasmids of essentially the same size in the LT-positive strain and only one in the LT-negative strain. It is possible that this additional plasmid encodes the LT.

The location of the LT genes on plasmid DNA has been tentatively confirmed by filter blot hybridization (4). It was possible by this technique to show homology between a *Bam*HI fragment carrying the LT genes from a porcine ST-LT plasmid (M. So, W. S. Dallas, and S. Falkow, manuscript in preparation) and one of the *Bam*HI fragments from the LT-positive O25:NM isolate. No homology was seen with the *Bam*HI digest of the plasmids from the LT-negative strain.

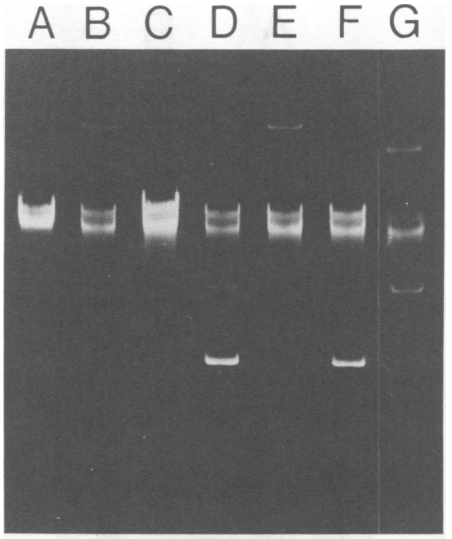

FIG. 2. *Agarose gel electrophoresis of E. coli K-12 resistant transconjugants. Lane A, E. coli K-12 recipient strain. Lanes B through D, Resistant transconjugants from mating with the toxigenic donor E2539C1. Lanes E and F, Resistant transconjugants from mating with the nontoxigenic donor E2539C3. Lane G, Toxigenic donor E2539C1.*

DISCUSSION

Several observations in this and other laboratories indicate that LT production is plasmid mediated. (i) Organisms other than the epidemic strain, *E. coli* serotype O25:NM, appeared to spontaneously lose toxigenicity. (ii) Not all *E. coli* O25:NM isolates were toxinogenic. (iii) An earlier report cited the instability of LT production in several genera of *Enterobacteriaceae* isolated during an epidemic of diarrheal illness (10). Unfortunately serotyping and ST production were not monitored at that time. (iv) The relative stability of heat-labile enterotoxinogenicity in this shipboard epidemic strain, under all storage conditions cited, is consistent with our earlier experience when an ST-producing *E. coli* serotype O78:H12 isolate was shown to harbor a 30-megadalton plasmid coding for toxinogenicity (25). A similar situation was observed during an outbreak of diarrhea at Crater Lake National Park in 1975 where LT-ST production was con-

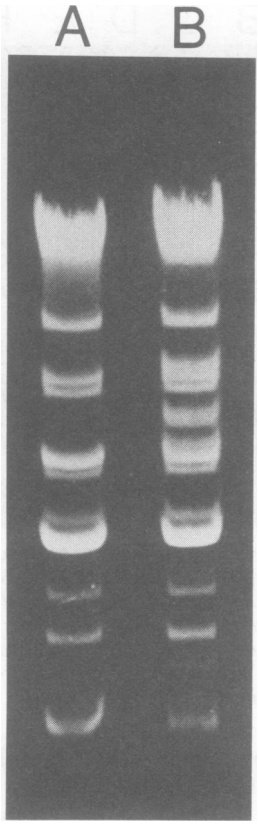

FIG. 3. *Restriction endonuclease BamHI banding pattern of plasmids from LT-positive and LT-negative O25:K98:NM isolates. Lane A, Nontoxigenic isolate E2539C3. Lane B, Toxigenic isolate E2539C1.*

fined to, and is stable to date in, *E. coli* serotype O6:H16 (15). Ørskov et al. have reported such serotype-toxigenicity stability in cultures collected internationally (14).

Our inability to conjugally transfer toxinogenicity in a one-step bacterial mating and the finding that toxigenic and nontoxigenic *E. coli* O25:NM isolates contained similar plasmid profiles in cleared lysates on agar gel electrophoresis first suggested that determinants for LT production might not be plasmid located. The chromosomal location of a cholera toxin gene (24) supported a potentially similar genetic control mechanism governing a toxin molecule (LT) of similar structure and function (3). Analysis of the nontoxigenic resistant transconjugant indicated that a transferable 57-megadalton R plasmid is either separate from an Ent plasmid which did not cotransfer at a detectable frequency or that the LT gene(s) is chromosomally mediated and not easily transferred conjugally. Incompatibility of the R plasmid and an Ent plasmid was also considered, but the observed long-term coexistence of the two properties in *E. coli* O25:NM suggests otherwise.

Further studies of *Bam*HI digests of plasmid DNA isolated from both R^+ Ent^- and R^+ Ent^+ *E. coli* O25:NM provided the evidence of an additional plasmid class in the Ent^+ strain. We feel that the possession of a region of homology with the LT-ST DNA sequence and its location on one of the restriction bands unique to the Ent^+ strain are strong evidence to suggest that the LT gene(s) is carried on a plasmid. Differences seen in Fig. 3 support this theory over the possible existence of one additional insertion sequence coding for LT. Recent data indicate that the epidemic strain also contains a prophage inducible to vegetative form by mitomycin C (23). This approach, which led to the transduction of toxigenicity, will facilitate further genetic analyses. The antibiotic resistance of nontoxigenic transconjugants suggests that these markers exist on separate plasmids.

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