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Conformity Between Heat-Labile Toxin Genes from Human and Porcine Enterotoxigenic *Escherichia coli*

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The genes encoding the heat-labile toxin of *Escherichia coli* were isolated by recombinant DNA methods from enterotoxigenic *E. coli* recovered from a human and a piglet with diarrhea. With restriction endonucleases, a fine-structure map was made for the toxin genes. Both genes were found to be highly homologous within the toxin-coding DNA, but the surrounding DNA sequences were found to be quite divergent. Analysis of in vitro-derived mutants demonstrated that the cistrons for the toxin proteins were located at the same sites on each restriction enzyme map.

Two reports have demonstrated immunological and structural differences between the heat-labile toxins from *Escherichia coli* of human (LTH) and porcine (LTP) origin (8, 10). Indeed, the relationship is reminiscent of that found between cholera toxin and LTP (3), and cholera toxin and LTH (9). However, the relationships among the genes encoding these toxins are different. Moseley et al. (14, 16) have shown that the LT gene (*elt*) isolated from an *E. coli* strain infecting piglets can be used in stringent hybridization conditions to detect the presence of *elt* in enterotoxigenic *E. coli* from human patients with diarrhea. This result is in contrast to the less stringent hybridization conditions required to detect DNA homology between porcine *elt* (elt_P) and the cholera toxin gene (15). The hybridization data showed that the LTH and LTP genes are more related to each other than to the cholera toxin gene. Reports on the structural and immunological uniqueness of LTH and LTP prompted the examination of the fine structure of two LT genes from strains for which LT had been purified and characterized. The results show that the two genes are virtually identical, suggesting that the immunological differences between LTH and LTP are most probably a reflection of only a few base pair (bp) differences that result in amino acid substitutions or, possibly, additions.

MATERIALS AND METHODS

Bacterial strains and plasmids. The human isolate of enterotoxigenic *E. coli* H74-114 was kindly provided by R. A. Finkelstein (19). The K-12 strain MM294 (endo I^- B_1^- r_k^- m_k^+) was used in all recombinant DNA experiments (12). The plasmid pBR322 confers ampicillin and tetracycline resistance to its host (2). The plasmid EWD030 is a recombinant plasmid derivative of pBR322 that codes for LTP and confers ampicillin resistance to its host (21).

Isolation of plasmids. Plasmid DNA from H74-114 was isolated as described previously by Portnoy et al. for 100-ml cultures (18). Further purification was obtained by cesium chloride centrifugation (final density, 1.55 g/ml) in the presence of 400 μg of ethidium bromide per ml for at least 3 h at 70,000 rpm and 20°C, using a Beckman VTi 70 rotor. Plasmid DNA from MM294 transformants was isolated by using a Triton X-100 cleared lysis method (21) coupled with the cesium chloride-ethidium bromide centrifugation procedure already described. Plasmid bands were removed from gradients by puncturing the side of the centrifuge tube with a 16-gauge needle with syringe. Ethidium bromide was removed by four extractions with an equal volume of water-cesium chloride-saturated isopropanol. Cesium chloride was removed, and the plasmid DNA was concentrated by ethanol precipitation.

DNA enzyme reactions and gel electrophoresis. All enzymes were purchased from Bethesda Research Laboratories, Gaithersburg, Md., and restriction enzyme and ligase reaction conditions were as described previously by O'Farrell et al. (17). Vertical agarose and polyacrylamide (19:1) slab gels were used to analyze restricted DNA samples (21). Gels were electrophoresed in Tris-acetate buffer. Southern blot hybridization procedures were carried out as previously described (6). The Bethesda Research Laboratories preparative electrophoresis system was used to isolate DNA fragments for molecular cloning. Approximately 14 μg of plasmid DNA from H74-114 was cut with *Pst*I and fractionated by the Bethesda Research Laboratories system. Fractions with human *elt* (elt_H) DNA sequences were identified by hybridization, using a minifold filtration manifold from Schleicher & Schuell Co., Keene, N.H. To denature the DNA, 20 μl of each 450-μl fraction was mixed with 180 μl of 0.5 N NaOH. After incubating for 5 min at room temperature, the solutions were transferred to nitrocellulose filter paper

using the manifold. The filter paper was next rinsed with 1 M Tris (pH 7) (200 μl per well) three times, followed by one wash with 1 M Tris (pH 7)–1.5 M NaCl. The filter paper was then treated in the usual fashion for Southern hybridization. The five hybridization-positive fractions were pooled and concentrated by ethanol precipitation. One-fifth of the sample was added to 1 μg of *Pst*I-cleaved pBR322, and T4 ligase was added. The final volume of the reaction mixture was 30 μl. Plasmid DNA was introduced into MM294 as described previously by Lederberg and Cohen (13). I isolated a recombinant plasmid, pWD600, composed of pBR322 and a DNA fragment with elt_H.

Mutations were introduced into pWD600 by using the following procedure (1). Plasmid DNA (10 μg) was cut with the appropriate restriction enzyme(s), and the ends of the DNA fragments were made flush (completely base-paired) by treatment with the Klenow fragment of DNA polymerase I as described by the supplier. Cleavage with *Xba*I left four bases unpaired at both 5′ ends of the duplex plasmid. In the presence of all four deoxyribonucleotides, the Klenow fragment catalyzed the addition of four bases at each end of the DNA. The net result was the addition of four extra nucleotide bps to the original DNA molecule. Conversely, *Sst*I-cut DNA had four bases unpaired at both 3′ ends of the duplex plasmid. The consequence of treatment of these molecules with the Klenow fragment was the loss of the four bases at each end of the DNA. In this instance, the net result was a loss of four nucleotide bps from the original plasmid. The ends of the DNA fragments were joined by T4 DNA ligase at a DNA concentration of 300 μg/ml. The rapid plasmid-screening procedure of Ish-Horowicz and Burke (11) was used to isolate small amounts of plasmid from transformants, and the DNA was then tested for the presence, absence, and number of diagnostic restriction enzyme sites.

Assays. Cultured Y-1 adrenal cells were used to assay LT activity as previously described (7). LT-B was assayed by the ganglioside immunosorbent assay (G_{M1}-ELISA) method described previously by Svennerholm and Holmgren (25). A ganglioside mixture from P-L Biochemicals, Inc., Milwaukee, Wis. was used in place of purified G_{M1}, and the detecting reagents were protein A-peroxidase (Zymed Laboratories, Burlingame, Calif.) and 4-aminoantipyrine (Sigma Chemical Co., St. Louis, Mo.)

RESULTS

Cloning *elt*. The molecular cloning of *elt* from a porcine source of enterotoxigenic *E. coli* has already been described (21). Briefly, the LT gene in this strain had been found to be a part of the 91,000-bp plasmid P307. elt_P was cloned as a 10,000-bp *Bam*HI DNA fragment into pBR322, forming the plasmid EWD030.

Since LTH from the human *E. coli* isolate H74-114 had recently been purified and characterized, it was decided to use this strain as a source of elt_H. To date, *elt* has only been found to exist in *E. coli* on extrachromosomal DNA. Therefore, the plasmid content of H74-114 was purified by using an alkaline lysis plasmid isolation method. H74-114 was found to carry two large plasmids of similar size (data not shown). The sizes of these plasmids were not determined. As has been previously reported, the presence of *elt* in a plasmid preparation could be detected by Southern blot hybridization, using a 770-bp *Hin*dIII DNA fragment from elt_P as a hybridization probe (6). Plasmid DNA from H74-114 was digested with two restriction enzymes, *Bam*HI and *Pst*I. *Bam*HI has been proven useful for cloning elt_P (21), whereas elt_H has been found to be within a single *Pst*I DNA fragment (26). The results of the hybridization experiment were that the H74-114 plasmid preparation contained DNA sequences homologous with the elt_P probe (data not shown). With either restriction enzyme, only a single DNA fragment was found to hybridize to the probe. Since *Pst*I generated the smaller of the two DNA fragments, this enzyme was used to clone elt_H.

The *Pst*I DNA fragment (5,450 bps) was first isolated by preparative gel electrophoresis, and fractions containing the hybridization-positive DNA fragment were identified by using a filtration manifold. The elt_H DNA fragment was then cloned into the *Pst*I site of pBR322. Transformants were screened for antibiotic phenotypes, and those strains that were resistant to tetracycline but sensitive to ampicillin (DNA inserted into the *Pst*I site inactivates ampicillin resistance) were analyzed further. A bacterial clone was identified that carried a hybridization-positive recombinant plasmid. Extracts from this strain were positive in the Y-1 adrenal cell test. The chimeric plasmid formed between *Pst*I elt_H from H74-114 and pBR322 was designated pWD600.

Restriction endonuclease map of *elt*. Fifteen different restriction endonucleases were used to analyze the cloned genes and the flanking DNA sequences (Fig. 1). (Two *Acc*I sites are shown on both restriction enzyme maps. Each *elt*-containing DNA fragment had more *Acc*I sites, but only those *Acc*I sites shown have been located.) The complete DNA sequence for elt_P has been determined (4, 24), and the position of this 1,350-bp gene within the 10,000-bp *Bam*HI fragment is shown in Fig. 1. The only region with multiple restriction enzyme sites in common between the two cloned DNA fragments was within *elt*. The restriction enzyme maps in Fig. 1 are arranged to emphasize this common region. This region included eight different restriction enzyme sites representing seven different enzymes (there were two *Hin*dIII sites). Double enzyme digests were used to further compare the restriction enzyme maps within *elt* (Fig. 2). At the top of the figure is a schematic diagram showing the common DNA region and the location of seven enzyme sites (*Acc*I is not

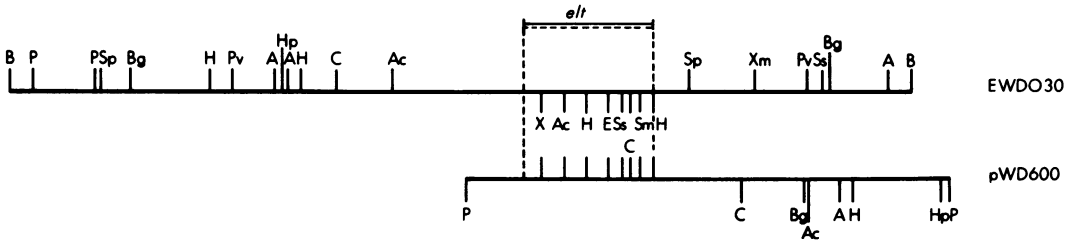

FIG. 1. Restriction endonuclease map of cloned DNA. EWD030 is a recombinant plasmid carrying elt_P, whereas pWD600 carries elt_H. The location of *elt* on EWD030 is shown. None of the cloning vector DNA is shown. Abbreviations for restriction enzymes are: B, *Bam*HI; P, *Pst*I; Sp, *Sph*I; Bg, *Bgl*II; H, *Hin*dIII; Pv, *Pvu*II; A, *Ava*I; Hp, *Hpa*I; C, *Cla*I; Ac, *Acc*I; X, *Xba*I; E, *Eco*RI; Ss, *Sst*I; Sm, *Sma*I; Xm, *Xma*III. The exact relative positions of restriction sites outside of *elt* that are closely clustered together (such as P-Sp and A-Hp-A in EWD030) were not determined. These enzyme recognition sites were mapped in reference to restriction sites within *elt*.

included). All DNA samples were first cleaved with *Xba*I, followed by digestion with a second enzyme (*Hin*dIII, *Eco*RI, *Sst*I, *Cla*I, or *Sma*I). The DNA fragments were then separated by electrophoresis through a 4% polyacrylamide gel. By using this method, the distances between the *Xba*I site and five different enzyme sites could be compared for the two cloned genes. Results from this type of analysis showed that DNA fragments with *elt* sequences were either the same size for the two cloned genes or slightly larger for the pWD600-derived DNA (migrated slower). Lanes A and B, of Fig. 2 are double digests with *Xba*I and *Hin*dIII. Fragment 1 was the *Hin*dIII DNA fragment that included the *Eco*RI, *Sst*I, *Cla*I, and *Sma*I sites and was the 770-bp fragment used in the Southern blot hybridization experiments. As is evident, pWD600 (Fig. 2, lane B) fragment 1 migrated more slowly than did EWD030 (Fig. 2, lane A) fragment 1. Fragment 2 in the same digest represented the DNA species with an *Xba*I end and a *Hin*dIII end. This fragment was the same size in both plasmids. The location of one *Hin*dIII site on each map (Fig. 1) has not been determined. However, it has been established by mapping experiments that this site was not within *elt*. In the case of pWD600, the additional site created a DNA fragment of 301 bps, whereas in EWD030 the fragment was 196 bps. In Fig. 2, lanes D through K, the DNA product of the double digestion that is relevant to the comparison is the smallest DNA fragment in each lane. The first lane in each pair is DNA from EWD030 (Fig. 2, lanes D, F, H, and J). With *Sma*I and *Cla*I as the second cutting enzymes, the slower mobility of the pWD600 DNA fragment was readily apparent (although the difference in migration was small). For *Sst*I and *Eco*RI, there appeared to be no difference in migration between the smallest fragments from pWD600 and EWD030.

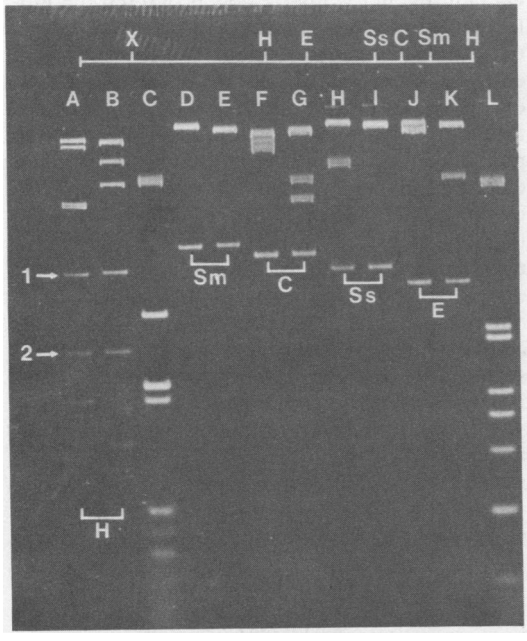

FIG. 2. Polyacrylamide gel of restricted DNA from EWD030 and pWD600. A 4% polyacrylamide gel was used to resolve DNA fragments. Above the gel is a schematic diagram showing restriction sites in elt_P. All samples were initially cleaved by *Xba*I, followed by cutting with a second enzyme. Lanes alternate with EWD030 and pWD600, beginning with EWD030 in lane A, except for lanes C and L in which pBR322 restricted with *Hae*II (lane C) or *Hin*fI (lane L) was used as a molecular weight marker. Abbreviations: X, *Xba*I; H, *Hin*dIII; E, *Eco*RI; Ss, *Sst*I; C, *Cla*I; Sm, *Sma*I. 1 and 2, Fragments 1 and 2.

Mapping the elt_H cistrons. Although the similarities between the restriction enzyme maps for both genes were convincing evidence that the two genes were essentially identical, it was important to demonstrate that the cistrons for the two toxin subunit proteins mapped to the same locus in elt_H as had been previously estab-

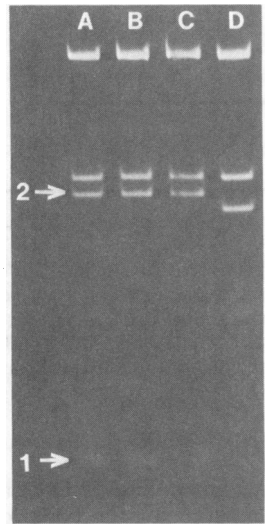

FIG. 3. Agarose gel of *Hin*dIII-cleaved plasmids. A 1.2% agarose gel was used to resolve DNA fragments from *Hin*dIII-cut pWD600 (lane A), pWD605 (lane B), pWD615 (lane C), and pWD601 (lane D). 1 and 2, Fragments 1 and 2.

lished for elt_P (4, 5, 24). By in vitro methods, mutations were introduced into pWD600, and the effect of each mutation on LT was determined after transformation into *E. coli* MM294. pWD600 was found to have single *Xba*I, *Sst*I, and *Sma*I sites, and these sites were used to make three different mutations in the plasmid (details are presented above). Mutant plasmid pWD601 had a deletion extending from the *Xba*I site to the *Sma*I site. This plasmid had lost the recognition sequences for *Xba*I, *Sst*I, and *Sma*I, and it also had a single *Eco*RI site (pWD600 had two *Eco*RI sites [data not shown]). Digestion with *Hin*dIII (Fig. 3) revealed that a *Hin*dIII site had also been lost and that the 770-bp fragment 1 was no longer present. As was predicted from the complete restriction map of pWD600, the migration of fragment 2 was also affected by the deletion. The unmapped 301-bp fragment was unaffected by the deletion. Another mutant plasmid was isolated in which the *Sst*I site of pWD600 was destroyed by deleting four internal bps of the recognition sequence. This plasmid, pWD605, was refractory to *Sst*I cleavage but had a *Hin*dIII digest pattern indistinguishable from pWD600, consistent with the presence of a small deletion. The third mutant plasmid, pWD615, was refractory to digestion with *Xba*I and was the result of a 4-bp insertion into the middle of the *Xba*I site. A *Hin*dIII digest of this plasmid was indistinguishable from pWD600 cut with *Hin*dIII, an observation which was consistent with the presence of a small insertion mutation.

The effect of each mutation on LT expression was measured by two assays: Y-1 adrenal cell and G_{M1}-ELISA (Table 1). The tissue culture assay was used to detect LT since the presence of both subunits is necessary for a positive response (5). The only positive bacterial supernatants were those carrying the wild-type recombinant plasmids EWD030 and pWD600. The presence of the LT-B subunit was assayed by G_{M1}-ELISA, an assay specific for LT-B. Sonic extracts from strains harboring EWD030 or pWD600 were positive in this assay, as were bacteria with pWD615 (Table 1). In contrast, the extracts from strains with pWD605 and pWD601 were negative in the G_{M1}-ELISA.

DISCUSSION

It has been shown that a DNA hybridization probe from elt_P can be used with fidelity to detect enterotoxigenic *E. coli* infecting both humans and piglets (6, 14, 16). These results suggested that all LT genes formed a homogeneous group irrespective of the isolation source of the infecting *E. coli*. Reports of immunological heterogeneity among LTs prompted the molecular cloning and detailed structural analysis of *elt* from a human isolate. When restriction enzyme maps of elt_H and elt_P were compared, every restriction site (eight sites) within elt_P was found to be present and in the same relative position in elt_H (Fig. 1). These results were in complete agreement with the previous hybridization experiments. However, when the relative distances between restriction sites within each gene were compared, a very small but consistent difference between elt_H and elt_P was noticed (Fig. 2). Some of the elt_H DNA fragments appeared larger than the corresponding segments of elt_P (Fig. 2, lanes A and B, fragment 1, lanes D and E, and F and G). Although the size differences were very small, the larger DNA fragments always included the 3′ end of *elt*, which has been shown to code for LT-B.

The size differences might be interpreted in several ways. The difference might be a conse-

TABLE 1. Phenotypes conferred by plasmids to MM294

Plasmid	Y-1 adrenal cells (LT-A, LT-B)[a]	G_{M1}-ELISA (LT-B)[b]
EWD030	+	+
pWD600	+	+
pWD601	−	−
pWD605	−	−
pWD615	−	+

[a] Both LT-A and LT-B are required for a positive response in Y-1 adrenal cells.

[b] Only LT-B is required for a positive G_{M1}-ELISA

quence of small differences in guanosine-cytosine content that subtly influence the DNA fragment migration rate. The nucleotide differences could lead to amino acid differences which, in turn, could be reflected in differences in amino acid compositions, as reported by Geary et al. (8). Of course, a nucleotide difference at a wobble position would have no effect on the amino acid composition. Alternatively, elt_H may indeed contain more nucleotides, and code for more amino acids, than does elt_P. This result is consistent with the apparent molecular weight of LT-BH, which is larger than LT-BP, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In contrast, DNA fragments corresponding to *elt*A (DNA encoding LT-A)-specific regions are the same size for both elt_H and elt_P (Fig. 2, lanes A and B, fragment 2, and lanes J and K). Again, these results are consistent with the apparent relative molecular weights of the A subunits, which are the same, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

It was also shown that the cistrons (*elt*A and *elt*B) for the two toxin subunits map to the same loci on the restriction enzyme map of both elt_H and elt_P. A deletion that extends from the *Xba*I site to the *Sma*I site would be expected, from the nucleotide sequence of elt_P, to delete most of *elt*A and *elt*B, leading to an LT^- phenotype. Table 1 shows that extracts from bacteria with the plasmid pWD601, which has a deletion in this region (Fig. 3), are negative in both the Y-1 adrenal cell assay and G_{M1}-ELISA. Both LT-A and LT-B subunits are necessary for a positive response in Y-1 adrenal cells, whereas G_{M1}-ELISA is an assay for LT-B. In this assay, the B subunit binds to the ganglioside mixture and is detected by antibody to LT (the vast majority of which is specific for LT-B). Extracts which were positive by G_{M1}-ELISA but negative in the Y-1 assay are deficient in LT-A. The *Sst*I site has been shown to be within *elt*B (4). A 4-bp deletion within the *Sst*I site would result in a frameshift mutation and loss of LT-B synthesis. A mutation procedure that could result in a 4-bp deletion was used, and the pWD600 derivative pWD605 was isolated. The loss of the *Sst*I restriction site and the *Hin*dIII DNA fragment pattern (Fig. 3) both suggested that a small deletion was present, although the exact nature of the deletion was not confirmed by DNA sequencing. Extracts of a strain carrying pWD605 were negative in both the Y-1 adrenal cell assay and G_{M1}-ELISA. Likewise, another frameshift mutation, this time in *elt*A, was predicted from adding four nucleotides at the *Xba*I site in pWD600 (24). The plasmid pWD615 was isolated from such an experiment. This plasmid had lost the *Xba*I recognition site, and again the *Hin*dIII DNA fragment pattern (Fig. 3) indicated that no gross plasmid alteration had occurred. (The exact nature of the mutation was not determined by sequencing.) As shown in Table 1, the phenotype conferred by this plasmid was LT^- (Y-1 adrenal cell) but $LT\text{-}B^+$ (G_{M1}-ELISA). These results are consistent with a mutation in *elt*A. All of these data suggest that the differences between elt_H and elt_P are due only to several bp changes or to additional nucleotides in *elt*B, or both. Final confirmation awaits DNA sequencing.

Interestingly, the restriction maps of the two *elt* DNA fragments were quite divergent with regard to the DNA sequences flanking the gene. Seven restriction sites were mapped to the right of elt_P (Fig. 1). None of these sites was present and in the same relative position in DNA to the right of elt_H. In pWD600, 650 bps of cloned DNA were present to the left of elt_H. *Pst*I was the only enzyme recognition site mapped to this region, but there was no homologous site present on the cloned DNA in EWD030. Given the high degree of homology between the two LT genes, it was curious that no homologous flanking DNA sequences were detected. Experiments are now in progress to determine the extent of relatedness between the flanking DNA sequences by a more sensitive method, Southern blot hybridization.

Finding almost total homology between elt_H and elt_P was expected. However, it was quite surprising that the DNA sequences flanking the gene appeared to be quite divergent, especially in light of the report of So et al. (20). These workers surveyed a small number of Ent (LT-bearing) plasmids and found that they constituted a homogeneous group with respect to nucleotide sequence, size, and mole fraction guanosine plus cytosine. Included in the study were isolates of both porcine and human origin. This evidence suggested that *elt* was probably confined to one plasmid type that was being transferred among various serotypes of *E. coli*. The distribution of *elt* was quite different from the ST gene, which was found to be a part of a very heterogeneous plasmid population and was later shown to be part of a transposon (22, 23). Recently, Yamamoto and Yokota (27) have presented evidence that at least one LT gene is flanked by repeated DNA sequences, which suggests that it may also be associated with a transposon. The mechanism by which *elt* has been spread among plasmids as well as the distribution of *elt* among different plasmid incompatibility groups remains to be determined. It will be interesting to determine the distribution of both elt_H and elt_P. It will be enlightening to determine if elt_P is confined to *E. coli* strains that infect piglets or if it also occurs in human

isolates. Likewise, the distribution of elt_H among *E. coli* isolates should be examined.

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