Cloning and Molecular Characterization of the B Subunit of *Escherichia coli* Heat-Labile Enterotoxin

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We have constructed a plasmid containing the gene for production of the B subunit of the heat-labile enterotoxin (LT-B) from a human isolate of *Escherichia coli*, strain H10407. The 0.8-kilobase gene fragment encoding synthesis of LT-B was cloned onto plasmid pBR322 after sequential digestion of the enterotoxin plasmid of strain H10407 with restriction endonucleases PstI and HindIII. LT-B was isolated by agarose affinity chromatography from cell lysates of recombinant clones expressing the B subunit. The B subunit was isolated in its oligomeric form, was structurally identical to native B subunit when examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, dissociated to monomeric B in the presence of 5 M guanidine, was immunologically identical to native B subunit in an enzyme-linked immunosorbent assay, and contained no demonstrable A subunit in any of the assays.

*Escherichia coli* produces diarrhea by a variety of mechanisms, including invasion of the intestinal epithelium or production of one or more enterotoxins. One of these enterotoxins, the heat-labile toxin (LT), has been purified and is known to be immunologically and physico-chemically related to the enterotoxin of *Vibrio cholerae* (choleragen) (4, 6, 18). Enterotoxigenic *E. coli* strains have been isolated from a number of different animals, including humans (9, 13, 14, 23–25), and recent investigations in a number of laboratories have revealed remarkable similarities between LTs isolated from strains of human and animal origin (11, 12, 16). Immunological differences between these have been noted, however, and we have recently demonstrated that these differences reside principally in the B subunit of the enterotoxin (5).

The enterotoxins of *E. coli* are plasmid mediated, and the plasmids that encode LT are large, at least 50 × 10⁶ daltons in mass (25). Extensive research has been performed to determine the nature of the genes encoding LT biosynthesis in both human and porcine isolates. Using recombinant DNA technology, So et al. (26) examined the LT genes of porcine strain P307 and identified a 5.8 × 10⁶-dalton *BamHI* restriction fragment containing all of the genes essential for LT synthesis. Subsequent work by Dallas et al. (8) and Dallas and Falkow (7) established the precise orientation of the genes for each of the subunits of LT (LT-A and LT-B) and confirmed previous observations (6) on the amino acid sequence homology between the B subunits of choleragen and of LT. The genes encoding LT biosynthesis in a human isolate, strain H10407, were identified by Yamamoto and Yokata (28), and the fragments specific for the A and B subunits were subsequently cloned onto separate carrier plasmids (29, 30).

Much of the emphasis of the previous work has been on the construction of a strain of *E. coli* which produces only LT-B for potential use as a live oral vaccine. Such a strain would be analogous to the strain of *V. cholerae* constructed by Honda and Finkelstein (15) which produces only the B subunit of cholera toxin. The nature of the B subunit produced by these clones that produce only B subunit has not been investigated beyond autoradiography of polyacrylamide gels prepared from immunoprecipitates of minicells carrying the recombinant plasmids.

We have recently demonstrated the efficacy of a vaccine for enterotoxigenic *E. coli* based on a synthetically derived heat-stable toxin (ST) cross-linked by carbodiimide coupling to the B subunit of LT isolated from a strain of porcine origin (17a). To facilitate the production of human LT-B as a component of the vaccine, we have constructed a recombinant plasmid containing the gene for production of the B subunit.

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only from a human isolate of *E. coli*, strain H10407. In this communication we describe the details of that construction and present data on the structural, functional, and immunological characteristics of the gene product so derived.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** The bacterial strains used were *E. coli* 711(10407), a K-12 transconjugant containing the ca. 60 × 10^6-dalton LT-ST plasmid of human enterotoxigenic isolate H10407 (5), and *E. coli* K-12 derivative MM294 *hsdR thy hsdM* + *enda* + (ATCC 33625). Plasmid pBR322, which codes for resistance to ampicillin and tetracycline, was obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.).

**Isolation of plasmid DNA.** Plasmid DNA was isolated by the procedure of Bolivar and Backman (1).

**Restriction endonuclease digestion.** Restriction endonucleases *PstI*, *EcoRI*, *HincII*, *Hinfl*, and *Avall* were products of Bethesda Research Laboratories. Reactions were carried out essentially as prescribed by the manufacturer.

**Electrophoresis.** Agarose gel electrophoresis was performed on 0.7% vertical slab gels in 0.04 M Tris-0.2 M sodium acetate-0.002 M EDTA (pH 7.8) (1). Phage λ DNA fragments generated by *HindIII* digestion were used as molecular weight standards. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the technique of Laemmli (19).

**Ligation.** Ligation reactions were carried out in 66 mM Tris-hydrochloride (pH 7.6)-6.6 mM MgCl₂-10 mM dithiothreitol-66 μM ATP. T4 DNA ligase (Bethesda Research Laboratories) was added to 10 μg of DNA, and the mixture was incubated at 4°C for 18 h. Where indicated, recircularization of the vector was reduced by treatment of the cloning vehicle, pBR322, with alkaline phosphatase conjugated to Sepharose (MATE-BAP; Bethesda Research Laboratories) before ligation. The reaction was performed in 10 mM Tris-hydrochloride (pH 8.0) at 65°C for 1 h.

**Transformation.** Transformation of *E. coli* MM294 was carried out as described by Bolivar and Backman (1). Cells were made competent by incubation in 30 mM CaCl₂ at 0°C for 20 min. Samples containing 0.2 ml of 10-fold-concentrated cells were added to DNA in 0.1 ml of cold ligation buffer supplemented with 30 mM CaCl₂ and incubated at 0°C for 1 h. The cells were then heated to 37°C for 2 min and held at room temperature for 10 min before being diluted into 4 ml of L broth. The cells were incubated at 37°C for 3 h, and transformants were selected on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) containing appropriate antibiotics. Fragments cloned into the tetracycline resistance gene of pBR322 were selected for by cycloserine enrichment before plating for selection (1). After 18 h of incubation in L broth containing 50 μg of ampicillin per ml, the culture was diluted 1:100 into fresh medium and supplemented with 4 μg of tetracycline per ml. After a 45-min incubation, 100 μg of o-cycloserine was added per ml, and incubation was continued for an additional 2 h. The culture was then centrifuged, and the pellet was resuspended in 20 ml of L broth. After 3 h of incubation, 0.1-ml aliquots were plated on Trypticase soy agar containing 50 μg of ampicillin per ml. A total of 96% of the clones obtained by cycloserine enrichment were ampicillin resistant (Ap') and tetracycline sensitive (Tc').

**Bioassay.** Assays in mouse Y1 adrenal cells were performed essentially as described by Sack and Sack (21).

**ELISA.** The enzyme-linked immunosorbent assay (ELISA) for LT was performed as previously described (17) except that the microtiter wells were precoated with 50 μg of type III gangliosides (Sigma Chemical Co., St. Louis, Mo.) per ml. This modification has been shown to increase the sensitivity of the assay 10-fold and is specific for compounds that bind to ganglioside and react with antiserum to LT. Clones to be assayed were cultured overnight in 20 ml of Trypticase soy broth (BBL), centrifuged, suspended in 2 ml of TEAN buffer (0.05 M Tris, 0.001 M EDTA, 0.003 M NaNO₃, 0.2 M NaCl [pH 7.5]) (2), and lysed by sonication. Lysates were clarified by centrifugation and serially diluted in phosphate-buffered saline (pH 7.4)-0.05% Tween 20.

**Purification of LT and LT-B.** The culture conditions and purification of LT and LT-B were as previously described (2, 3). Organisms were cultured overnight in 10 liters of Evans medium (10) at 37°C with vigorous aeration and agitation after inoculation with 10⁶ viable bacteria per ml. The bacteria were harvested by centrifugation at 4°C, and the cells were suspended in TEAN and lysed by sonication. The crude lysate was clarified by centrifugation and precipitated with 60% saturated (NH₄)₂SO₄. The precipitate was then harvested and dialyzed against TEAN and LT or LT-B purified by agarose affinity chromatography (4).

**Enterotoxin subunits.** Analysis of the A and B subunits of LT and of LT-B was performed by gel filtration under dissociating conditions as described previously (6).

**Antiserum.** Antiserum to LT-B was prepared by immunizing a goat with 1 mg of guanidine-purified LT-B (6) suspended in 2 ml of Freund complete adjuvant. Antiserum to the A subunit of cholera toxin was prepared as previously described (2).

**Guidelines used for recombinant DNA experiments.** The experiments reported here were performed using PI-EK1 conditions as specified in the Guidelines for Recombinant DNA Technology published by the National Institutes of Health.

**RESULTS**

**Isolation of pDF82.** *E. coli* 711(10407) is an LT⁺ST⁺ transconjugant. This strain was derived by phenotypically tagging the enterotoxin plasmid of *E. coli* H10407 by transposition from an *F‘* is let::Ts5 plasmid and conjugally transferring the Ts5-tagged plasmid to *E. coli* K-12 strain 711 (5). The 10407 plasmid was purified, cut with the restriction endonuclease *PstI*, and ligated with T4 DNA ligase to pBR322 which had been cut with *PstI* and treated with alkaline phosphatase. The ligation mixture was used to transform *E. coli* MM294, and Ap' Tc' transformants were identified (185 of the 6,484 Tc' transformants screened were Ap'). These trans-
formants were assayed for LT production by using the Y1 adrenal cell system and ELISA. Plasmid DNA was isolated from several LT+ transformants and analyzed on 0.7% agarose gels. One isolate, pDF82, contained only a single plasmid (Fig. 1, lane A) and was positive in both the Y1 assay and ELISA. When recut with PstI, this plasmid contained only two fragments, one corresponding to the 4.2-kilobase (kb) cloning vector, pBR322, and one of 5.2 kb coding for synthesis of LT (data not shown). Subsequent analysis with PstI, EcoRI, HindIII, HincII, HindII, and AvaI confirmed the size of this fragment and the absence of internal PstI sites.

Isolation of pDF87. The cloned LT DNA region was recloned from pDF82 into the single HindIII site in the tetracycline resistance gene of pBR322. pDF82 and pBR322 were cut with HindIII, mixed, and joined by T4 DNA ligase. The ligation mixture was again transformed into E. coli MM294, and Ap′Tc′ transformants were identified after cycloserine enrichment for tetracycline sensitivity. The transformants were assayed for loss of LT biological activity on Y1 adrenal cells and for production of LT-B antigen by ELISA. Plasmid DNA was isolated from transformants negative in the Y1 assay and positive in ELISA and analyzed. pDF87 (Fig. 1, lane C) was cut by HindIII into two fragments, one identified as pBR322 and a smaller one (0.8 kb) coding for synthesis of LT-B (Fig. 1, lane D). Significantly, the 1.5-kb HindIII fragment (Fig. 1, lane B) that codes for production of the A subunit of LT (30) is absent from pDF87.

Production and characterization of LT and LT-B. A major consideration in the construction of a clone producing LT-B only was that the B subunit still be responsive to existing purification schemes, such as agarose affinity chromatography (4). Both LT (from pDF82) and LT-B (from pDF87) were purified by this technique. Crude preparations were individually applied to separate columns of Sepharose 4B, and, after a TEAN wash, the columns were eluted with 0.2 M galactose in TEAN. In each case, a single peak absorbing at 280 nm eluted just before the leading edge of the applied galactose.

The contents of each peak were examined by SDS-PAGE (Fig. 2). The peak eluted by galactose from pDF82 (Fig. 2, lane A) contained two bands, one representing the oligomeric B subunit (upper band) and one representing the A subunit. The peak eluted by galactose from pDF87 (Fig. 2, lane B) contained only the oligomeric B subunit (upper band). The band corresponding to the A subunit (lane A, lower band) was not present in pDF87 (lane B). SDS-PAGE was performed in 7.5% tube gels by the technique of Laemmli (19). Each tube contained 10 μg of protein as determined by the method of Lowry et al. (20). The band near the bottom of each gel (TD) is the tracking dye.

FIG. 1. Agarose gel electrophoresis of partially purified lysates of pDF82 (lane A), HindIII-cleaved pDF82 (lane B), pDF87 (lane C), and HindIII-cleaved pDF87 (lane D). Plasmid DNA was prepared by the procedure of Bolivar and Backman (1) and examined by electrophoresis in 0.7% agarose slab gels (0.04 M Tris, 0.2 M sodium acetate, 0.002 M EDTA [pH 7.8]).

FIG. 2. Analytical discontinuous electrophoresis in SDS-PAGE of agarose affinity-purified preparations from pDF82 (lane A) and pDF87 (lane B). Both preparations contained a band corresponding to oligomeric B subunit (upper band). The band corresponding to the A subunit (lane A, lower band) was not present in pDF87 (lane B). SDS-PAGE was performed in 7.5% tube gels by the technique of Laemmli (19). Each tube contained 10 μg of protein as determined by the method of Lowry et al. (20). The band near the bottom of each gel (TD) is the tracking dye.
subunit (lower band). As expected, the peak eluted by galactose from pDF87 (Fig. 2, lane B) contained only a single band representing the oligomeric B subunit. Even when the gels were overloaded with as much as 100 μg of protein, no band corresponding to the A subunit was visible in SDS-PAGE.

Each peak was then examined by gel filtration under dissociating conditions. Figure 3 shows the chromatographic profile of each preparation when applied to a Sephadex G-75 column in the presence of 5 M guanidine. The profile from pDF82 showed two peaks, the first corresponding to the A subunit and the second corresponding to the monomeric B subunit of LT. In contrast, pDF87 contained only a single peak corresponding to the monomeric B subunit. Thus, pDF87 produced only the B subunit of LT, and that subunit was structurally indistinguishable from LT-B produced as a component of the LT holotoxin.

When assayed by ELISA against antiserum to LT-B and antiserum to cholera toxin subunit A, pDF82 was shown to produce holotoxin which reacted with both subunit antisera (Fig. 4). LT-B produced by pDF87 was immunologically identical to that produced by pDF82, but did not contain antigen which was recognized by antiserum to cholera toxin subunit A. No demonstrable A antigen was present, even at 10 μg, an amount 4,000-fold-higher than the limit of resolution in this assay (data not shown).

DISCUSSION

We have constructed a clone of E. coli capable of producing LT-B only and have successfully purified B subunit from that clone by agarose affinity chromatography. Others have cloned the gene fragments for LT-A and LT-B onto a variety of plasmid vehicles (7, 22, 29, 30), but analysis of the gene products was limited to autoradiography of polyacrylamide gels prepared from immunoprecipitates of minicells carrying the recombinant plasmids. It had not been demonstrated, for example, that the cloned B subunit retained the ability to bind the agarose, that it was assembled into its oligomeric form when constructed de novo in the absence of the A subunit, and that it was immunologically and structurally identical to native B subunit.

In constructing a clone which produces LT-B, we used as a recombinant vehicle the plasmid pBR322. In addition to unique restriction sites for PstI and HindIII, pBR322 contains two partially overlapping promoters, one of which (P1)
is able to transcribe DNA inserted into the HindIII site in the tetracycline resistance region (27). This would be particularly important since the B gene contains no identifiable promoter region and presumably is transcribed from the A subunit promoter in vivo (8). After sequential digestion of the enterotoxin plasmid of human isolate H10407 with PstI and HindIII and transformation of E. coli MM294, a clone was selected which was negative in the mouse Y1 adrenal tumor cell assay and positive in ELISA for B antigen. Analysis by agarose gel electrophoresis revealed only a single plasmid consisting of pBR322 and a 0.8-kb HindIII fragment coding for LT-B. These findings are consistent with those of Yamamoto et al. (30), who inserted a 0.77-kb HindIII fragment of isolate H10407 into plasmid pACYC177, and of Sanchez et al. (22), who recently reported the construction of an LT-B recombinant plasmid by insertion of a 0.8-kb HindIII fragment from pEWD299 (8), derived from porcine strain P307, into the plasmid vehicle pACYC184.

It is not possible to predict whether the gene product of our clone is identical to that produced by other clones constructed elsewhere. Diverse results are often obtained when a particular gene fragment is inserted into different cloning vehicles or into different sites on the same vehicle (27). Our intent was to construct a clone that would produce significant amounts of LT-B that could be purified by existing procedures. The nature of the galactose-binding region of the B subunit is unknown, but alterations in structure resulting from deletion or gene fusion could interfere with that binding and hamper purification. Since galactose binding is presumably a reflection of the affinity of LT for its native receptor, GM1, the use of the ganglioside-ELISA for selection of antigen-producing clones eliminated any in which the galactose-binding capability of the B subunit was significantly impaired. Likewise, minor alterations could have had a profound influence on immunological determinants within the molecule. LT-B produced by this clone was immunologically indistinguishable from native B subunit.

LT-B produced in this manner has considerable potential as a component of a vaccine designed to stimulate immunity against entero-toxigenic E. coli. We have shown (17a) that B subunit can be cross-linked to synthetically derived ST and that this immunogen evokes a four- to sevenfold increase over control values of serum immunoglobulin G and mucosal secretory immunoglobulin A antitoxin levels to each of the component toxins, thus providing significant (P < 0.001) protection against either toxin or heterologous serotypes of viable strains which produce these toxins. The construction of a clone of E. coli which produces only LT-B allows the production of B subunit under conditions which are relatively rapid, less expensive than existing methods, and, more importantly, free of contaminating subunit A.

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LITERATURE CITED