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Isolation and Nucleotide Sequence Determination of a Gene Encoding a Heat-Stable Enterotoxin of *Escherichia coli*

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A gene encoding a heat-stable enterotoxin (ST) from an *Escherichia coli* strain isolated from a human with diarrhea was cloned and characterized by nucleotide sequence analysis. The gene was found to be partially homologous to a previously characterized ST gene from an *E. coli* strain of bovine origin. Hybridization studies showed that most ST-producing strains of *E. coli* isolated from humans with diarrhea possess genes highly homologous to either the ST gene from the bovine strain or the ST gene characterized in the present study.

Enterotoxigenic *Escherichia coli* (ETEC) strains, pathogenic for humans and animals, produce two classes of plasmid-mediated enterotoxins. The heat-labile toxin (LT) is of high molecular weight; consists of two subunits; is functionally, structurally, and genetically related to the enterotoxin of *Vibrio cholerae*; and is highly homologous (though not identical) among different strains of LT-producing *E. coli* (6, 18). Its mechanism of action involves the activation of adenylate cyclase (9). The heat-stable toxin (ST) is of low molecular weight and acts by stimulating guanylate cyclase (10).

Biological, biochemical, and genetic evidence suggests that STs from different strains of *E. coli* represent a heterogeneous group of toxins. There are at least two distinct types of ST (4, 12). ST I (also referred to as STa) is methanol soluble and is active in the infant mouse model. ST II (also referred to as STb) is methanol insoluble; it is not active in the infant mouse model, but can be detected in ligated pig ileal loops. Plasmid genes encoding ST I have been isolated by recombinant DNA methods (8, 27). One of these, designated ST Ia (26), was initially found in a bovine *E. coli* strain; it has been shown to be part of a bacterial transposon and to be genetically distinct from ST II (29), and its nucleotide sequence has been determined (30). An isolated fragment of DNA from a porcine *E. coli* strain which encodes a portion of the ST Ia molecule was utilized as a genetic probe in a study designed to identify ETEC from patients with diarrhea by colony DNA hybridization (21). Although all LT-producing strains were identified by an LT gene probe, many ST-producing strains (as determined by the infant mouse assay) did not possess DNA homologous to the gene encoding ST Ia, suggesting the existence of a heterologous gene encoding ST I. Similar results were subsequently reported by So et al. (26), who designated the heterologous toxin(s) ST Ib. Preliminary nucleotide sequence data of a possible ST Ib gene have been reported (8).

This study describes the isolation and sequence determination of a gene encoding ST Ib from a human isolate of *E. coli* and the use of the isolated ST Ib gene as a probe for the detection of ETEC in patients with diarrhea.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* 153837-2 produces ST and was isolated by us from the diarrheal stool of a human in Bangladesh. *E. coli* C600 and HB101 are *E. coli* K-12 strains that have been described previously (2, 3). Plasmids pBR322 and pWD299, a recombinant plasmid encoding LT production, have been described previously (3, 6). pRIT10036 is a recombinant plasmid encoding ST production and had been reported previously under the designation CLS-2 (21); it was constructed by the insertion of approximately 1.1 megadaltons of DNA from a porcine strain of *E. coli* into plasmid vector pBR322 (13). Plasmid pSLM004 is described in the present study.

Plasmid DNA isolation. Plasmid DNA was isolated as described by So et al. (28).

Restriction of DNA. Restriction endonucleases were obtained from New England Biolabs, Inc., Boston, Mass. The digestion of DNA by these enzymes was carried out under the conditions specified by the supplier.

Ligation and transformation of DNA. Bacteriophage T4 DNA ligase was a gift from Walter Dallas, Cetus Corp., Berkeley, Calif. Plasmid pBR322 DNA (approximately 0.01 μg) was cleaved with the appropriate

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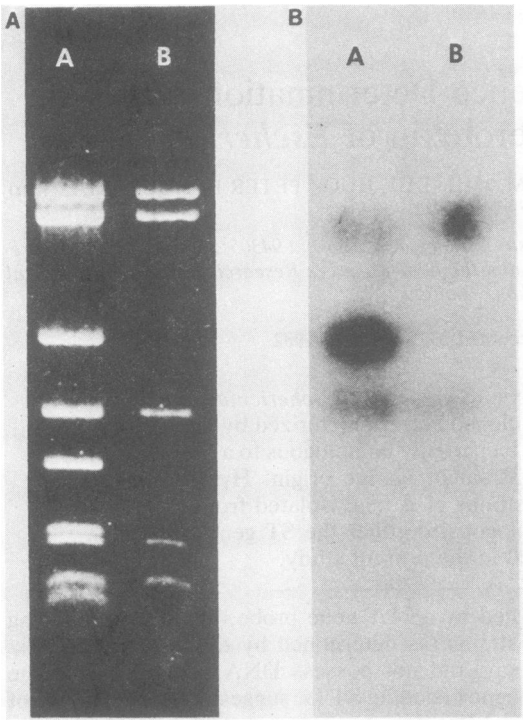

FIG. 1. Partial homology between ST Ia and ST Ib genes. Plasmid DNA was digested with *TaqI*, transferred to nitrocellulose, and hybridized to the ST Ia probe under conditions of reduced stringency. (A) Agarose gel electrophoresis of *TaqI*-digested plasmid DNA. (B) Autoradiograph of blot after hybridization to ST Ia probe. Lanes A, pSLM003; lanes B, pBR322.

restriction endonuclease and mixed with the appropriate DNA fragments to be ligated at an approximate ratio of 1 pBR322 molecule per 100 DNA fragments. The ligation was carried out in a reaction volume of 10 μl at 0°C for 2 h.

Ligation reaction conditions were 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 5 mM NaCl, 10 mM dithiothreitol, and 40 mM ATP. The entire ligation mixture was used to transform *E. coli* K-12 HB101 by the following protocol: 2 ml of an overnight stationary culture of HB101 in L broth was diluted into fresh L broth and incubated for 100 min at 37°C with agitation. The cells were washed once in 20 ml of 10 mM $CaCl_2$–10 mM morpholinepropanesulfonic acid (MOPS; pH 6.5; Sigma Chemical Co., St. Louis, Mo.). The cells were suspended in 2 ml of 75 mM $CaCl_2$–10 mM MOPS (pH 6.5)–0.5% glucose. A ligated DNA mixture was then added, and cells were kept on ice for 45 min. The transformation mixture was then incubated at 42°C for 10 min. A volume of 0.5 ml of fresh L broth was added, and the mixture was incubated for 2 h at 37°C. The mixture was then plated on media containing the appropriate antibiotic to select for transformants.

Southern blot analysis of restricted DNA. The procedure of Southern (31) was used with modifications (20). Plasmid DNA (0.1 μg) was digested with *TaqI*, and the resulting fragments were separated by electrophoresis in 2% agarose. The DNA was transferred to nitrocellulose and hybridized to radio-labeled probe in a low-stringency hybridization solution consisting of 25% formamide, 5× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate, and 0.02% each of Ficoll (molecular weight, 400,000), polyvinyl pyrrolidone (molecular weight, 360,000), and bovine serum albumin. The nitrocellulose was incubated in the hybridization solution without a probe for 3 h at 37°C and then transferred to fresh hybridization solution containing approximately 10^6 cpm of heat-denatured probe DNA per ml and 75 μg of sheared, heat-denatured calf thymus DNA per ml. The hybridization was incubated overnight at 37°C. The nitrocellulose was then washed in 5× SSC–0.1% sodium dodecyl sulfate for 45 min at 54°C, transferred to 2× SSC for 30 s at room temperature, and allowed to dry at room temperature. The nitrocellulose was then exposed to Kodak X-Omat R film (Eastman Kodak, Rochester, N.Y.) with a single Cronex Lightning-Plus intensification screen (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) for 24 h at −70°C. The film was developed by the manufacturer's instructions.

Nucleotide sequence analysis. The methods of Maxam and Gilbert (15) and Sanger et al. (23) were used for nucleotide sequence analysis. For the Maxam-Gilbert procedure, restriction fragments to be sequenced were separated by electrophoresis in 10% polyacrylamide slab gels, removed from the acrylamide by electroelution, and purified by DEAE-cellulose chromatography (DE-52, Whatman) (25). The 5′ termini were labeled with [γ-^{32}P]ATP (New England Nuclear Corp., Boston, Mass.) by a T4 polynucleotide kinase (Bethesda Research Laboratories, Rockville, Md.) reaction (15). The 3′ ends of *HpaII* fragments were labeled with [α-^{32}p]dCTP by an *E. coli* DNA polymerase-Klenow fragment (Boehringer Mannheim Corp., Indianapolis, Ind.) reaction. The fragment (5 pmol) was incubated with 25 μCi of labeled nucleotide (3,000 Ci/mmol), 1 nmol of unlabeled dGTP, and 2 U of Klenow fragment in a reaction buffer consisting of 6 mM Tris (pH 7.4), 6 mM $MgCl_2$, and 50 mM NaCl. The reaction volume was 25 μl. The reaction was incubated at room temperature for 30 min and heated at 65°C for 10 min, and the DNA was precipitated with ethanol.

For the Sanger procedure, a *HpaII* fragment to be sequenced was cloned into bacteriophage M13 mp8 (17). The primer DNA was a gift of Staffan Normark, University of Umea, Umea, Sweden. The sequencing strategy is described below.

Isolation of ETEC from patients with diarrhea. Stools were obtained from 108 persons sequentially admitted to the intravenous rehydration ward of the International Centre for Diarrhoeal Disease Research, Bangladesh. These patients were routine admissions for diarrhea with moderate to severe dehydration, and no attempt was made to select patients with probable ETEC infections. Stools were spotted onto nitrocellulose for colony hybridization and streaked onto MacConkey agar for the isolation of colonies. Two colonies and a pool of five colonies were cultured for appropriate standard enterotoxin assays (16).

Colony DNA hybridization. The preparation of DNA fragments specific for LT and ST genes (probe DNA) has been described previously (20, 21). The LT probe

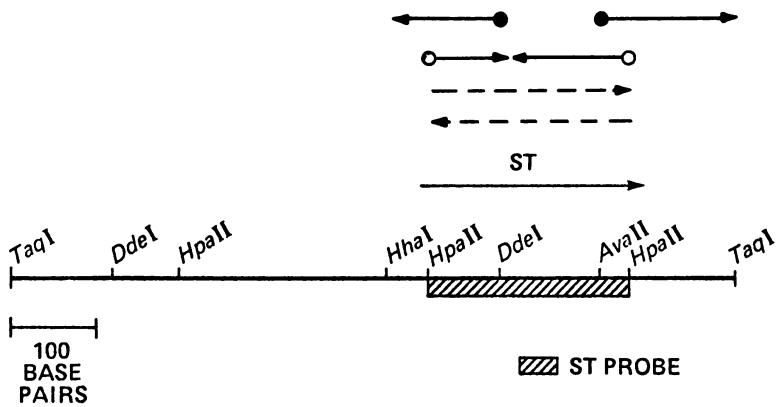

FIG. 2. Restriction map of inserted DNA in pSLM004. The fragment used as a hybridization probe is shown, and the position and orientation of the gene are indicated by the solid arrow labeled ST. The sequencing strategy is indicated at the top of the figure. Broken lines indicate fragments sequenced by the Sanger technique (23) from DNA inserted in both orientations in bacteriophage M13 (17). Solid lines indicate fragments sequenced by the technique of Maxam and Gilbert (15). Open circles designate 3'-labeled ends, whereas closed circles indicate 5'-labeled ends.

consisted of a 1,200-base-pair *Hin*cII fragment from pWD299 encoding the LT-A subunit (19). The ST Ia probe was a 157-base-pair *Hin*fI fragment from pRIT10036 (20). ST Ib probe DNA consisted of a 215-base-pair *Hpa*II fragment of pSLM004. This fragment was obtained by the cleavage of pSLM004 DNA with *Eco*RI + *Hin*dIII, the isolation of the ST gene-containing fragment by electrophoresis, the cleavage with *Hpa*II, and the subsequent isolation of the appropriate fragment. The probe DNA was labeled with α-^{32}P-deoxynucleotides (New England Nuclear) by nick translation (14) to a specific activity of approximately 5×10^7 cpm/μg.

Colony hybridization with growth from spotted stools was performed as previously described (21) with modifications. The initial procedures were performed at Dacca. Nitrocellulose disks (90 mm, BA-85; Schleicher & Schuell Co., Keene, N.H.) were placed on MacConkey agar and inoculated with a loopful of each diarrheal stool to be tested. After overnight incubation at 37°C, the nitrocellulose was removed from the agar and placed on a piece of Whatman no. 3 paper saturated with 0.5 N NaOH for 10 min. The nitrocellulose was then transferred three times to a piece of Whatman paper saturated with 1.0 M ammonium acetate–0.02 N NaOH for 1 min. After the three 1-min transfers on the same buffer, the nitrocellulose was kept on buffer-saturated Whatman paper for 10 min, removed, and allowed to dry at room temperature. The nitrocellulose was then incubated at 65°C overnight. The disks were transported to Bangkok, where probe preparation, hybridization, and autoradiography were performed at the Armed Forces Research Institute for Medical Sciences. Five to ten processed nitrocellulose filters were immersed in a high-stringency hybridization solution (identical to the low-stringency solution described above, except that the concentration of formamide was raised to 50%). Preincubation and hybridization were as described for the Southern blot analysis, except that the concentration of probe DNA was reduced to 10^5 cpm/ml. The disks were washed in 5× SSC–0.1% sodium dodecyl sulfate for 45 min at 65°C, rinsed in 2× SSC at room temperature, and allowed to dry at room temperature. Autoradiography was performed as described for Southern blot analysis.

Assays for enterotoxin production. The infant mouse assay (7) was used for the detection of ST production. The Chinese hamster ovary (CHO) cell assay (22) was used for the detection of LT.

RESULTS

Construction of a recombinant plasmid expressing ST Ib. Purified plasmid DNA from *E. coli* 153837-2 was cleaved with restriction endonuclease *Bam*HI, and the resulting fragments were ligated to *Bam*HI-cleaved plasmid pBR322 DNA. The ligation mixture was used to transform *E. coli* HB101. Ampicillin-resistant transformants were screened for sensitivity to tetracycline, and ampicillin-resistant, tetracycline-sensitive colonies were screened for production of ST by the infant mouse assay. One transformant was found to produce ST. The restriction analysis of plasmid DNA from this strain revealed that a *Bam*HI fragment of approximately 1.9 kilobase pairs had been inserted into pBR322. This recombinant plasmid was designated pSLM003.

Demonstration of partial homology between the genes encoding ST Ia and ST Ib. The existence of ST Ib was demonstrated by the failure of ST Ia gene probes to detect homologous DNA sequences in some ST I-producing strains of *E. coli* (21, 26). These hybridization studies were carried out under conditions of high stringency, so that only highly homologous DNA was detected. pSLM003 was examined for partial ho-

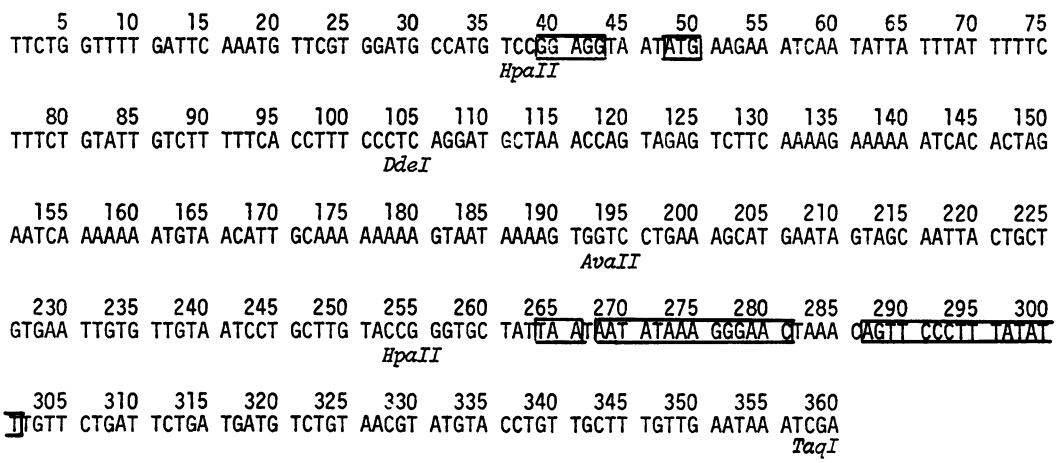

FIG. 3. Nucleotide sequence of the gene encoding ST Ib. The sequence of the sense strand is shown, reading from 5' to 3'. Boxed sequences are, in order, the ribosome binding site, the start codon, the stop codon, and the region of dyad symmetry suggestive of a transcription termination sequence.

mology to a gene encoding ST Ia by hybridization under conditions of reduced stringency, allowing the detection of sequences of incomplete homology. The ST Ia probe was radiolabeled with ^{32}P and hybridized to *Taq*I-restricted pSLM003 DNA which had been separated by electrophoresis in a 2% agarose slab gel and transferred to nitrocellulose (Fig. 1). Among the inserted sequences from strain 153837-2, partial homology was detected between the ST Ia probe and a specific *Taq*I fragment of pSLM003 of approximately 840 base pairs. Apparent homology with pBR322-specific fragments was due to a low level of contamination of the probe preparation with some pBR322 sequences.

Nucleotide sequence of the gene encoding ST Ib. The 840-base-pair *Taq*I fragment containing partial homology with the ST Ia probe was recloned into the *Cla*I site of pBR322. This plasmid was designated pSLM004. *E. coli* HB101 (pSLM004) was found to produce ST as detected in the infant mouse assay. The ST-encoding *Taq*I fragment of pSLM004 was mapped with regard to specific cleavage by several restriction endonuclease enzymes by the analysis of partial and double digests (Fig. 2). Four *Hpa*II fragments within the cloned *Taq*I DNA insert were isolated, spotted on nitrocellulose, and hybridized to the ST Ia probe at low stringency. A *Hpa*II fragment of approximately 215 base pairs displayed partial homology to the ST Ia probe (data not shown). This fragment and portions of the adjacent fragments were subjected to nucleotide sequence analysis according to the strategy shown in Fig. 2. The results are shown in Fig. 3. The structural gene is preceded by a ribosomal binding site (GGAGG) (24) beginning at position 39, followed by a translational start codon (ATG) beginning at position 48. The coding region of the gene ends with the translational terminational signal, TAA, beginning at position 264. The termination signal is followed by a 15-base-pair region of dyad symmetry from positions 268 to 301, suggestive of a transcriptional termination structure in the ST Ib gene transcript (Fig. 4).

Use of an ST Ib gene probe for detection of ETEC in patients with diarrhea. Diarrheal stools from 108 patients were screened for the presence of ETEC by standard assays and by hybridization with LT, ST Ia, and ST Ib gene probes. The results are shown in Fig. 5 and Table 1.

The LT probe detected ETEC in all stools from which LT-producing colonies were isolated, as well as from 11 additional stools that failed to yield LT-producing *E. coli* as assayed by standard techniques. Thirty-six patients were found to be infected with ST-producing ETEC by the infant mouse assay. Stools from 34 of these patients yielded bacterial growth possessing DNA sequences homologous to one or both of the ST I DNA probes. The probes detected homologous DNA in bacterial growth from the stools of four patients from which ST-producing ETEC were not detected by the infant mouse assay. Of the the 38 stools that were positive for ST by colony hybridization, 3 were detected with the ST Ia probe, 33 were detected with the ST Ib probe, and 2 yielded bacterial growth with DNA sequences homologous to both ST probes.

DISCUSSION

The present study has confirmed the existence of heterologous genes encoding ST I and reports the isolation and nucleotide sequence determination of one of these genes from a strain of *E.*

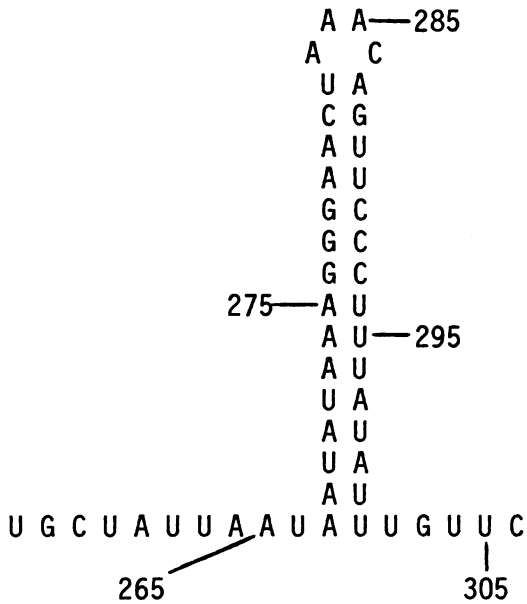

FIG. 4. Possible secondary structure of transcript of 3' end of ST Ib gene. Such structures are associated with the termination of transcription (1).

coli of human origin. The sequence of the ST Ib gene isolated in the present study is shown in Fig. 6, with the predicted amino acid sequence of its product in comparison with the nucleotide sequence of the ST Ia gene and the amino acid sequence of its product as reported by So and McCarthy (30) and the amino acid sequence of an ST I from a human strain determined by Chan and Giannella (5). Although the two nucleotide sequences appear to share a common evolutionary origin, significant divergence has taken place. There are no insertions or deletions; all changes are substitutions. Among the 216 nucleotides from codons 1 to 72, there are a total of 67 nucleotide differences between the ST Ia and ST Ib genes, resulting in a sequence divergence of 31%. These changes result in a total of 29 amino acid changes, with most changes (16 of 29) being nonconservative (based on conservation of polarity and charge).

Despite the nucleotide sequence divergence observed among the genes encoding ST Ia and ST Ib, their products show a great deal of structural similarity. The analysis of the ST Ia gene product suggests that the initial protein is processed for transmembrane transport (13). The nucleotide sequence of ST Ia supports this interpretation, predicting that 13 of the first 18 amino acids are hydrophobic and that this portion of the molecule is related to other signal peptides involved in the transmembrane transport of a protein (30). A similar structure is predicted for the ST Ib product with 13 of the first 19 amino acids being hydrophobic. Another similarity is the number and position of cysteines. The identical position of seven cysteines in the two gene products suggests that similar intramolecular associations may take place in the two molecules, resulting in similar secondary structures. The greatest degree of homology between the two genes occurs between codons 53 and 68, suggesting that this region may play an essential role in the function of the molecule. This interpretation is supported by the existence of the 18-amino acid-peptide (5, 22), with ST

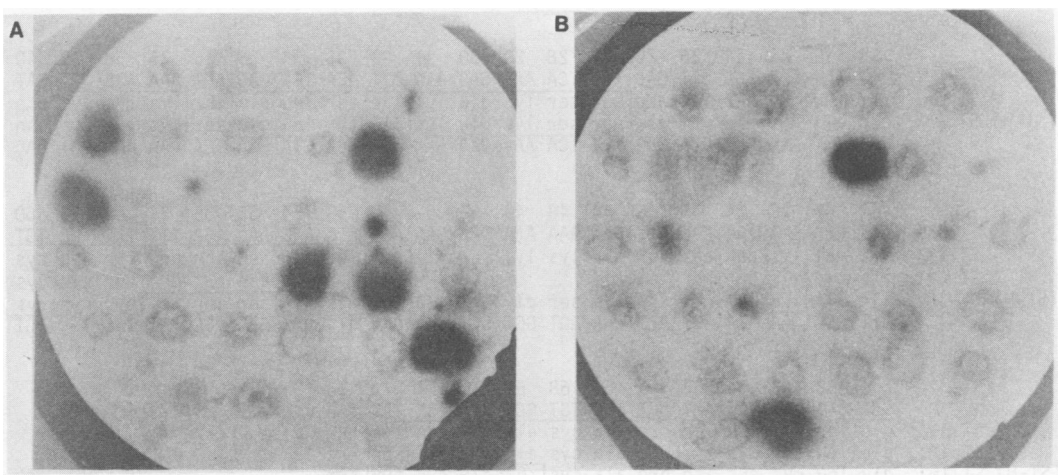

FIG. 5. Example of colony hybridizations. Thirty stools from patients with diarrhea were inoculated onto duplicate nitrocellulose filters on MacConkey agar. The processed filters are shown overlaid with the resulting autoradiographs after the exposure of the hybridized filters. (A) Hybridization with LT probe; (B) a duplicate filter hybridized with the ST Ib probe.

TABLE 1. Detection of ETEC by colony DNA hybridization

Toxin produced[a]	No.	Detected by probe[b]			
		LT	ST Ia	ST Ib	0
LT	3	3	0	1	0
ST	24	6	3	20	2
LT + ST	12	12	2	11	0
0	69	5	0	3	61

[a] Two colonies and a pool of five colonies from each stool were tested for LT production by the CHO cell assay and for ST by the infant mouse assay.

[b] A loopful of each stool was spotted on three nitrocellulose filters overlaid on MacConkey agar for colony hybridizations with each of the three probes.

activity corresponding to codon positions 55 to 72 (Fig. 6). It is of interest to note that this region also encodes a sequence of six amino acids (codons 63 to 68) homologous with conotoxins, 13- to 15-amino-acid-long toxic peptides purified from the venom of the marine snail *Conus geographus* (11). Computer analysis of the amino acid sequences of ST I and conotoxins reveals this homology to be significant (M. So, personal communication).

The enterotoxin gene probes detected ETEC in the stools of 37 of 39 patients (95%) from whom ETEC was isolated, as well as in eight stools from which no ETEC was isolated by standard techniques. Reconstruction experiments suggest that ETEC/normal flora *E. coli* ratios as low as 1/100 may be detected by the colony hybridization technique described here (unpublished data), thereby resulting in a somewhat higher sensitivity than the standard procedure at the International Centre for Diarrhoeal Disease Research, Bangladesh (assaying two colonies and a pool culture of five colonies from each stool). Also, a number of stools found to contain ETEC producing only ST by standard assays were found by the colony hybridizations to contain *E. coli* with DNA sequences homologous to the ST and LT gene probes. Our past experience with isolated strains of *E. coli* suggests that these results are most likely due to false-negative results with the CHO cell assay. In a previous study (21), such discrepancies were always resolved on retesting by the CHO assay. We have not isolated an *E. coli* which possesses DNA sequences homologous to the LT probe yet did not produce LT. ST-producing *E. coli* were isolated from two stools in which no homologous sequences to either ST I probe were detected. Whether these results are due to false-positive infant mouse assays or, in fact, suggest the existence of yet another class of ST genes is not yet known.

The preliminary data reported here regarding the use of the ST Ib gene as a probe for the detection of ETEC suggest that most ST-producing *E. coli* strains which are not detected by

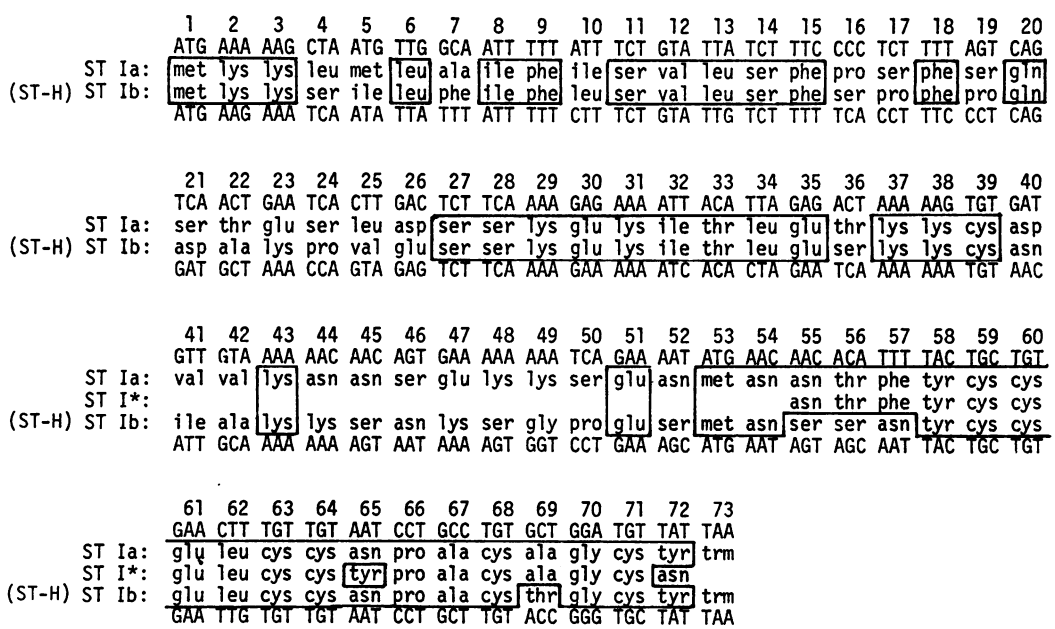

FIG. 6. Comparison of nucleotide and amino acid sequences of ST Ia, ST Ib, and an 18-amino-acid peptide (designated here ST I*) with ST I activity. The ST Ia sequences are from So and McCarthy (30). The ST I* sequence was reported by Chan and Giannella (5). Boxed regions indicate regions of amino acid homology.

the ST Ia gene probe are, in fact, detected by the ST Ib gene probe. The proportion of strains hybridizing with the ST Ia gene probe differs significantly from the results of a similar study done in the same location 1 year previously and also from the proportion of strains detected by this probe among a number of isolates from Morocco (21). The previous study found that approximately two thirds of strains producing only ST possess sequences homologous to the ST Ia probe, whereas very few strains producing ST and LT hybridize with the probe. The present study indicates that the majority of all ST-producing strains of human origin in Bangladesh possess DNA sequences homologous to the ST Ib gene probe. These results and a further investigation of the use of enterotoxin gene probes for the detection of ETEC (19) suggest that the distribution of the two genes may differ in different geographical locations and may change with time. This emphasizes the potential usefulness of the enterotoxin gene probes in the study of the epidemiology of enterotoxin-encoding genes, plasmids, and strains.

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