JOURNAL OF BACTERIOLOGY, Nov. 1987, p. 5201–5208
0021-9193/87/115201-08$02.00/0

Vol. 169, No. 11

Fusion of *Escherichia coli* Heat-Stable Enterotoxin and Heat-Labile Enterotoxin B Subunit

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Received 12 March 1987/Accepted 14 August 1987

The 3′ terminus of the DNA coding for the extracellular *Escherichia coli* heat-stable enterotoxin (ST) devoid of transcription and translation stop signals was fused to the 5′ terminus of the DNA coding for the periplasmic B subunit of the heat-labile enterotoxin (LT_B) deleted of ribosomal binding sites and leader peptide. By RNA-DNA hybridization analysis, it was shown that the fused DNA was transcribed in vivo into an RNA species in close agreement with the expected molecular weight inferred from the nucleotide sequence. The translation products of the fused DNA resulted in a hybrid molecule recognized in Western blots (immunoblots) with antibodies directed against the heat-labile moiety. Anti-LT_B antibodies coupled to a solid support bound ST and LT_B simultaneously when incubated with ST-LT_B cellular extracts. By [^{35}S]cysteine pulse-chase experiments, it was shown that the fused ST-LT_B polypeptide was converted from a precursor with an equivalent electrophoretic mobility of 20,800 daltons to an approximately 18,500-dalton species, which accumulated within the cell. The data suggest that wild-type ST undergoes at least two processing steps during its export to the culture supernatant. Blocking the natural carboxy terminus of ST inhibited the second proteolytic step and extracellular delivery of the hybrid molecule.

Enterotoxigenic *Escherichia coli* strains have been isolated from 9 to 30% of cases of diarrheal disease in infants (12). Two kinds of plasmid-encoded enterotoxins have been shown to induce secretory diarrhea in humans and animals: the heat-stable (ST) and the heat-labile (LT) families of enterotoxins (32, 37). ST subtype A (ST_A) is an extracellular (13), methanol-soluble, 18- to 19-amino-acid peptide; due to its low molecular weight, it is not naturally immunogenic. The genetic information encoded by ST DNA results in the extracellular delivery of the ST enterotoxin; the amino acid sequence inferred from the DNA sequence (11, 27, 38; H. Stieglitz, R. Robledo, R. Fonseca, L. Cervantes, L. Covarrubias, F. Bolivar, and Y. M. Kupersztoch, manuscript in preparation) shows an initial 19-amino-acid region that has the properties of a leader peptide (31), followed by a 53-amino-acid-long peptide that includes the active 19-amino-acid ST enterotoxin (2) thought to be decisive for the final localization of the toxin. The gene is followed by proposed translation and transcription termination signals (27, 38). The periplasmic LT holotoxin (30) is composed of one A subunit (molecular weight [MW] 25,500) and five B subunits (LT_B, MW 11,500) (11). LT_B is the receptor(s)-binding moiety (28) and by itself is not toxic. LT_B alone, as well as the LT holotoxin (14, 30), is localized in the periplasm.

LT is thought to be translated from a single mRNA (8, 39); from the nucleotide sequence, ribosomal binding sites for *toxA* and *toxB* have been inferred. Downstream from the *toxB* gene in the transcribed RNA, regions of dyad symmetry likely to be involved in transcription termination have been identified (43).

To explore whether the 5′-terminal ST-encoding DNA could provide the structural information necessary for mobilization of the periplasmic LT_B to the exterior of the cell and to generate a peptide with both ST and LT_B epitopes, a fused ST-LT_B gene was constructed. In this report, we present the construction of such hybrid DNA and its in vivo transcription and translation products. We show that strains harboring the fused DNA produced both ST and LT_B. It is also shown that the fused ST-LT_B is first detected as a precursor which after chasing is accumulated as a cell-associated form apparently 2,300 daltons smaller. The data support the hypothesis that the ST enterotoxin undergoes two proteolytic steps during its export process. The results are discussed in relation to the export of ST and the use of the fusion product for immunoprotection against both toxins and for their detection.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *E. coli* strains used were JM83 [*ara* Δ(*lac-pro*) *rpsL thi* φ80d Δ*lacZM15*] and HB101 (F$^-$ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ$^-$).

Plasmid pRIT10250 (M. DeWilde, M. Ysebeart, and N. Harford, p. 596 *in* S. B. Levy, R. C. Clowes, and E. L. Koenig, ed., *Molecular Biology, Pathogenicity and Ecology of Bacterial Plasmids* [Plenum Publishing Corp., New York]) was kindly supplied by N. Harford; it is pACYC184 (5) with a 600-base-pair (bp) *Eco*RI ST$^+$ fragment obtained from strain 20590 (an *E. coli* strain isolated in Bangladesh from a patient with diarrhea). Plasmid EWD299, supplied by W. Dallas, is pBR313 with a DNA insert that encodes the LT enterotoxin derived from a porcine *E. coli* strain (8). Plasmid pUC13 was described by Messing (26). pGK40 has an *Eco*RI-*Hin*dIII DNA fragment derived from EWD299 ligated to the same sites of pUC8 (8). pGK22 was obtained by inserting a 600-bp ST$^+$ *Eco*RI fragment, purified from pRIT10250 DNA, into the *Eco*RI site of pUC8; the orientation of the ST gene is the same as the *lacZ* gene. Plasmids pGK26 and pGSK51 were derived from pUC13 and are described in the Results section. Plasmids pGP1-2 and pT7-3 have been described (41) and were kindly provided by S. Tabor. Plasmid pYK153 was obtained by ligating the *Eco*RI-*Hin*dIII (ST-LT_B DNA fragment) shown in Fig. 1, section 5, to pT7-3 DNA digested with the same enzymes; in this plasmid, the ST-LT_B DNA is downstream from the T7 promoter of plasmid pT7-3 in front of and in the same

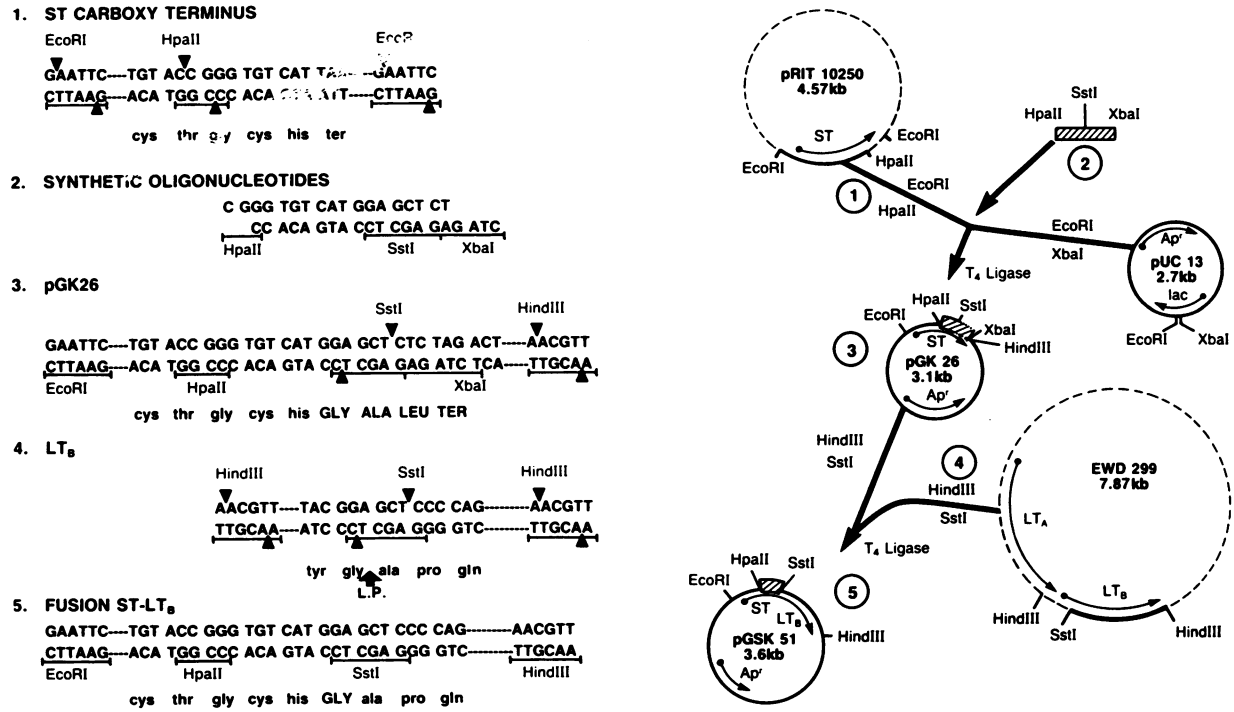

FIG. 1. Construction of the ST-LT_B fusion. (Left) Relevant nucleotides. The restriction sites are underlined. Digestions with enzymes in the subsequent cloning steps are shown as solid triangles. The inferred wild-type amino acid sequences are shown in lowercase letters below the DNA sequence; capital letters are modifications. L.P., Site of action of the leader peptidase on LT_B [4]. (Right) Solid lines mark the DNA fragments used in the next cloning step. The box represents the synthetic oligonucleotides. The arrows inside the circles show the direction of transcription, and the solid dot shows ribosomal binding sites. A 390-bp ST-encoding DNA fragment obtained by *Eco*RI-*Hpa*II digestion of pRIT10250 [1] was incubated with 5'-phosphorylated preannealed synthetic oligonucleotides [2] and with *Eco*RI-*Xba*I-cleaved pUC13 DNA. The mixture was ligated and used to transform strain JM83, selecting for Ap^r white (Lac^-) transformants on L agar supplemented with ampicillin and Xgal. The resulting plasmid, pGK26 [3], was isolated, digested with *Hin*dIII and *Sst*I, and ligated to a 505-bp LT_B-encoding DNA fragment of plasmid EWD299, obtained by digestion with the same restriction endonucleases [4]. Ap^r transformants were selected on L agar supplemented with ampicillin. The resulting plasmid, pGSK51 [5], harbored the fused ST-LT_B DNA.

orientation as the β-lactamase gene. L (22) and T (24) media were used when indicated. The pulse-chase experiments were done in C broth (33) modified as follows: 2 g of NH_4Cl, 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 3 g of NaCl, 0.018 g of $MgCl_2$, 6 g of yeast extract, and 3% glycerol in 1 liter of solution. This medium was supplemented with the minimal amino acid requirement of strain HB101 (proline [200 μg/ml] and leucine [100 μg/ml]). When indicated, the medium was supplemented with ampicillin (100 μg/ml), tetracycline (12.5 μg/ml), kanamycin (100 μg/ml), and/or the chromogenic substrate for β-galactosidase, Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (40 μg/ml).

Isolation of plasmid DNA and restriction endonuclease analysis. Large-scale (500-ml cultures) and small-scale (5-ml cultures) plasmid isolations were accomplished by lysis with NaOH-sodium dodecyl sulfate (SDS) after lysozyme spheroplasting of the cultures as described by Maniatis et al. (23). Supercoiled plasmid DNA was separated from linear and open circular DNAs by ethidium bromide-CsCl isopycnic density centrifugation (18). DNA was digested with restriction endonucleases following the recommendations of the manufacturers. Restriction enzyme analysis of DNA fragments was done by agarose or polyacrylamide gel electrophoresis, and the bands were developed by incubating the gels with ethidium bromide and visualizing their fluorescence under UV light (23). When necessary the DNA restriction fragments were recovered from the gels by excising the fluorescent bands of interest and recovering the nucleic acids in an electroeluter (International Biotechnologies, Inc.) as suggested by the manufacturer.

Transformation. Bacteria were transformed by the $CaCl_2$-RbCl method described by Kushner (19).

DNA sequencing. The sequence of the two synthetic oligodeoxyribonucleotides was confirmed by the chemical cleavage method of Maxam and Gilbert (25). The hybrid ST-LT_B DNA was sequenced by the dideoxy- chain-terminating method described by Sanger et al. (34).

RNA isolation and Northern blot (RNA) hybridization analysis. Total cellular RNA was isolated from cells grown in 100 ml of L broth to a density of 2×10^8 to 5×10^8 cells per ml at 37°C following the procedure described by Aiba et al. (1) modified as follows: 2×10^{10} to 5×10^{10} cells were pelleted by centrifugation and suspended in 3 ml of lysis buffer (0.02 M sodium acetate, pH 5.5, 1 mM disodium EDTA, 0.5% SDS). This suspension was extracted two times with 3 ml of 60°C phenol equilibrated with 0.02 M sodium acetate (pH 5.5). The RNA was precipitated from the aqueous phase by the addition of 3 volumes of absolute ethanol, suspended in 3 ml of lysis buffer, and ethanol precipitated twice. The resulting pellet was suspended in 1 ml of distilled water.

The RNA species were fractionated by agarose gel electrophoresis (4 to 5 h, 125 V) in a 1.8% gel containing formaldehyde (2.2 M) in a 0.04 M MOPS (morpholinepropanesulfonic acid, pH 7.0)-based buffer as described by Maniatis et al. (23). The gel was blotted to a nitrocellulose membrane, which was then dried and hybridized against the

20-mer oligomer TTACAACACAATTCACAGGA ST probe previously radiolabeled by incubation with polynucleotide kinase and [γ-^{32}P]ATP. Hybridization was at 42°C for 14 h (23), after which the filter was washed four times at room temperature in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) containing 0.5% SDS, followed by washing at 46°C in 2× SSC containing 0.1% SDS. The dried filter was exposed to Kodak XOmat AR film, and the film was developed.

Western blot protein-antibody analysis. Sonic extracts of bacteria harvested from 20-ml cultures were prepared as described previously (13), and the proteins were fractionated by SDS-polyacrylamide gel electrophoresis in a 0.1% SDS–20% polyacrylamide separating gel (20). The proteins were electrophoretically transferred to nitrocellulose membranes and incubated for 15 h at 42°C with a solution of 10% low-fat dry milk (15), 0.01% thiomersal in phosphate-buffered saline [(PBS) 0.137 M NaCl, 0.016 M Na_2HPO_4, 0.002 M KH_2PO_4]. The membrane was rinsed with PBS and washed two times for 30 min each with PBS containing 0.05% Tween 20, followed by a 4-h room temperature incubation with goat antibodies raised against an LT toxin purified from an *E. coli* strain isolated from a human patient (goat anti-LT), a gift of R. Finkelstein (10), diluted 1:1,000 in PBS-Tween containing 0.1% bovine serum albumin. The filter was washed three times for 20 min each with PBS-Tween. The membrane-bound goat anti-LT was detected by an enzyme-linked immunosorbent assay (ELISA) by incubating the membrane in a 1:2,000 dilution of horseradish peroxidase coupled to rabbit anti-goat immunoglobulin (Cappel Laboratories) in PBS-Tween-bovine serum albumin at 4°C overnight. The membrane was washed three times for 20 min each with PBS-Tween, and to enhance the signal a third antibody, mouse anti-goat immunoglobulin G (IgG) conjugated to horseradish peroxidase (Cappel Laboratories) diluted 1:2,000 in PBS-Tween, was added to the membrane, incubated for 2 h at room temperature, and washed under the conditions described for the first antibody. The color was developed by incubating in PBS (pH 7.4) containing 4-chloro-1-naphthol (3 mg/ml) and 0.1285 μl of H_2O_2 per ml. The antibodies used in this assay were preadsorbed three times at 4°C for 1 h with 1 volume of a mixture (1:1) of a PBS suspension of intact bacteria and a sonic extract of strain HB101(pUC13). The suspensions were centrifuged, and the supernatant was used in the assay.

Determination of ST and LT_B. The ST assay was done as previously described (9, 24) by injecting intragastrically four newborn Swiss albino mice per sample and, after 4 h of incubation, determining the ratio of intestine weight to body weight minus intestine. The LT_B activity was determined by a ganglioside-promoted ELISA in microtiter plates as described previously (40) and by immunodiffusion in an Ouchterlony test (6) with goat anti-LT.

Immunoadsorption procedure. The B subunit of LT was purified from sonic cellular extracts of strain HB101(pGK40) as described (7), applying the extracts to a Sepharose 4B column (Pharmacia Fine Chemicals) and washing the column with TEAN (50 mM Tris, 1 mM disodium EDTA, 3 mM NaN_3, 200 mM NaCl, pH 7.5). The effluent was monitored at 280 nm, and when the optical density (OD) returned to the baseline, LT_B was eluted with 500 mM galactose in TEAN. The first OD_{280} peak, five fractions before it, and five after it were tested for LT_B by the Ouchterlony test (6). The positive fractions were pooled, dialyzed, concentrated by ultrafiltration with a UMO5 membrane (Amicon Corp.), and analyzed in SDS-polyacrylamide gel electrophoresis; staining the gel with silver detected a single band of 11,500 daltons that confirmed the purity of LT_B. The purified LT_B was used to immunize New Zealand rabbits by injecting 100 μg subdermically and by boosting with 50 μg after 1 month. Immune serum was prepared from the blood of these animals by standard methods. Total serum IgG was purified with protein A-Sepharose CL-4B (Pharmacia Fine Chemicals) following the recommendations of the manufacturers, binding the serum to the column, washing with 20 mM phosphate buffer (pH 8.5), and eluting the IgG with 50 mM citrate buffer, pH 3.5. The purified IgG was bound to activated agarose (Reacti-Gel 6X; Pierce Chemical Co.) at pH 8.5 in 0.1 M borate buffer for 36 h, followed by blocking the active groups by the addition of 1.0 M ethanolamine at pH 9.0 for 3 h at room temperature, as suggested by the manufacturer.

Pulse-chase experiment and analysis of radioactive peptides. Strains HB101(pGP1-2)(pT7-3) and HB101(pGP1-2)(pYK153) were grown separately at 29°C on C broth (supplemented with ampicillin and kanamycin) to 0.5 OD_{595} units; the cultures were shifted to 42°C, and 15 min later rifampin (200 μg/ml) was added and incubation was continued for an additional 10 min. The cultures were shifted back to 29°C, and after 25 min, radioactive [^{35}S]cysteine (15 μCi/ml) was added to each suspension; C medium prewarmed to 29°C and supplemented with cold cysteine (8 mg/ml) was added 1 min after the pulse, and immediately a 2-ml sample was removed and added to 4°C stop solution (to yield a final concentration of 33 μg of chloramphenicol, 133 mM sodium azide, and 6.6 mM 2,4-dinitrophenol). At 2, 5, 15, and 60 min, portions were removed and added to an ice-cold stop solution. The bacteria were pelleted by centrifugation and suspended in 50 μl of ice-cold TEAN, immediately followed by the addition of 50 μl of lysis buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.04% bromophenol blue). The samples were boiled for 5 min and fractionated by SDS-polyacrylamide gel electrophoresis in a 17% acrylamide gel (20). After electrophoresis, the gel was stained with Coomassie blue, and the radioactive bands were fluorographically detected with sodium salicylate.

Biochemical assays and enzymes. The methods of Lowry et al. (21) and Bradford (3) were used to quantify protein concentration. RNA concentrations were determined by the method of Mejbaum as described by Schneider (35). Restriction endonucleases, T4 DNA ligase, and polynucleotide kinase were purchased from Boehringer Mannheim Biochemicals. Radioisotopes were from New England Nuclear Corp.

RESULTS

Construction of ST-LT_B. The fused ST-LT_B DNA was designed to include the ST DNA region coding for the transcription initiator and regulatory elements, the coding region of the 53 amino acids that precede the extracellular ST, and the active ST devoid of transcription and translation stop signals. This DNA fragment was to be followed by the LT_B-encoding DNA devoid of ribosomal binding sites and its signal peptide, but preserving the LT_B transcription and translation stop signals. Figure 1 illustrates the steps followed to accomplish this fusion at the nucleotide level and the corresponding plasmids. Strain JM83(pGSK51) was shown to harbor the fused ST-LT_B DNA. The expected and inferred nucleotide and amino acid sequences are shown in Fig. 1, section 5; it illustrates that at the fusion site, the original ST sequence was preserved, with the introduction of glycine between the last amino acid of ST (histidine) and the

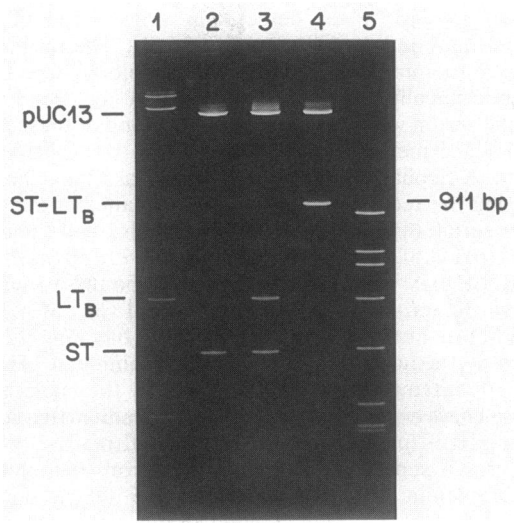

FIG. 2. Polyacrylamide gel electrophoresis of restriction endonuclease-digested plasmid DNAs. Lanes: 1, EWD299 (LT) DNA digested with *Hin*dIII and *Sst*I; 2, pGK26 (ST) DNA digested with *Eco*RI and *Sst*I; 3, pGSK51 (ST-LT_B) DNA digested with *Eco*RI, *Sst*I, and *Hin*dIII; 4, pGSK51 DNA digested with *Eco*RI and *Hin*dIII; 5, MW markers (pBR322 DNA treated with *Alu*I).

first amino acid of the mature LT_B (alanine). The inferred translation product was expected to have the two toxin peptides in the proper reading frame.

Restriction nuclease analysis and nucleotide sequence of the fused ST-LT_B DNA. To demonstrate that the expected restriction fragments were present in the fused DNA, pGSK51 DNA was isolated and digested with *Eco*RI, *Hin*dIII, and/or *Sst*I. Digestion with *Eco*-RI-*Hin*dIII (Fig. 2, lane 4) resulted in a 2,686-bp and a 911-bp band; the former fragment had the electrophoretic mobility of the cloning vehicle (pUC13) digested with the same enzymes, and the latter band had the mobility expected for the fused ST-LT_B DNA. Digestion of pGSK51 DNA with *Eco*RI, *Sst*I, and *Hin*dIII resulted in three distinct bands (Fig. 2, lane 3); the largest corresponded to pUC13 DNA, the 505-bp band had the same mobility as the LT_B *Hin*dIII-*Sst*I fragment of EWD299 (Fig. 2, lane 1), and the 406-bp band had the same mobility as the ST *Eco*RI-*Sst*I DNA fragment of pGK26 (Fig. 2, lane 2). Thus, the expected restriction sites and fragments (Fig. 1, section 5) were present in pGSK51; the fused DNA was excised by the digestion with *Eco*RI and *Hin*dIII, and the two moieties were separable by the *Sst*I site (Fig. 2, lanes 4 and 3), as expected from the design of the fusion (Fig. 1, section 5). To further prove that the designed fusion resulted in the expected nucleotide sequence, pGSK51 was cleaved with *Eco*RI and *Cla*I. The *Cla*I site is localized 88 bp downstream from the *Sst*I site shown in Fig. 1, section 5. The *Eco*RI-*Cla*I fragment was cloned into M13mp18 previously treated with *Eco*RI-*Acc*I. Single-stranded phage DNA was isolated and sequenced as described in Materials and Methods. The sequenced DNA confirmed that the hybrid had the expected nucleotide sequence at the fusion site (Fig. 1, section 5).

Transcription of the fused ST-LT_B DNA. We examined the ST regulatory transcriptional elements and whether transcription stop signals were generated as a consequence of the fusion. To analyze the in vivo transcription of the fused ST-LT_B DNA, total RNA was isolated from strain HB101 harboring plasmids pGSK51 (ST-LT_B), pGK22 (ST only), pUC13 (cloning vector), and pGK26 (ST plus three amino acids; Fig. 1, section 3). The RNA was fractionated in agarose gel electrophoresis, and the gel was blotted to nitrocellulose and hybridized against a ^{32}P-labeled oligonucleotide probe complementary to a region near the 3′ hydroxy terminus of the ST mRNA (Fig. 3). When the source of the RNA was a wild-type ST^+ strain (lane 2), a 270-nucleotide (nt) band was detected by the probe. Strain HB101(pGSK51) carried the fused DNA; analysis of the RNA isolated from this strain showed a 630-nt band detected by the ST probe (Fig. 3, lane 3); this size is very close to that expected for the 671-nt ST-LT_B mRNA calculated for a transcript initiated from the ST promoter (L.-M. Guzman-Verduzco, K. James, and Y. M. Kupersztoch, manuscript in preparation) (216 nt for ST, 315 nt for LT, plus 140 nt proposed for the localization of the transcriptional termination loop of LT_B [57]). RNA isolated from strain HB101 (pGK26) showed a wide RNA band that hybridized to the probe (Fig. 3, lane 4); very likely the decreased mobility and spread of this mRNA band reflect elimination of the transcription stop signals that resulted from cloning the ST-derived DNA fragment to yield plasmid pGK26 (Fig. 1, section 3). When the RNA was from strains harboring the LT holotoxin or a plasmid containing the LT_B subunit, no hybridization was detected by the ST probe (data not shown). Thus, the fused ST-LT_B DNA was transcribed into RNA species that agreed closely with the expected size.

Translation of the fused ST-LT_B DNA. In vivo translation of the ST-LT_B hybrid was examined by fractionating total sonic extracts of bacteria by SDS-polyacrylamide gel electrophoresis, blotting the gel to nitrocellulose, and detecting the bands reacting to an antibody directed against LT_B (Fig. 4). When HB101(pGK22), an ST-producing strain, was analyzed, anti-LT_B antibody was not bound (Fig. 4, lane 1). When the sonic extracts were from HB101(pGK40), an

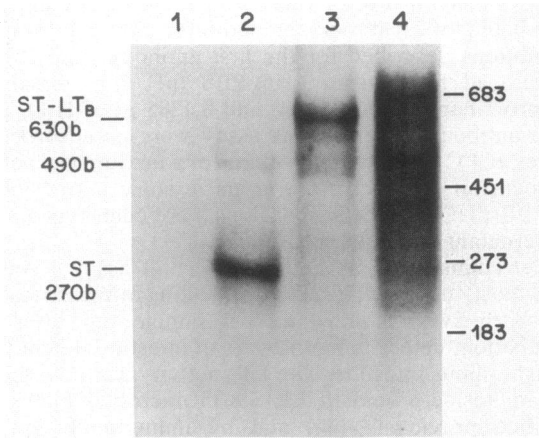

FIG. 3. Autoradiography of Northern blot of total bacterial RNA. RNA (20 μg) isolated from each strain was fractionated by agarose gel electrophoresis in MOPS-formaldehyde buffer, blotted to nitrocellulose paper, and hybridized against a ^{32}P-labeled synthetic 20-mer oligonucleotide complementary to the conserved region of the inferred ST mRNA. The host strain was HB101 harboring the following plasmids: lane 1, pUC13, the cloning vehicle; lane 2, pGK22, a wild-type ST DNA fragment cloned into the vehicle; lane 3, pGSK51, the hybrid ST-LT_B DNA cloned in pUC13; lane 4, pGK26, an ST^+ DNA encoding three more amino acids at the carboxy terminus of ST and lacking the ST transcriptional termination signal. MW markers were obtained by cleaving pSP64- and pSP65-derived plasmids with suitable restriction endonucleases, followed by incubation with SP6 RNA polymerase in the presence of [^{32}P]ATP. Sizes are shown in bases.

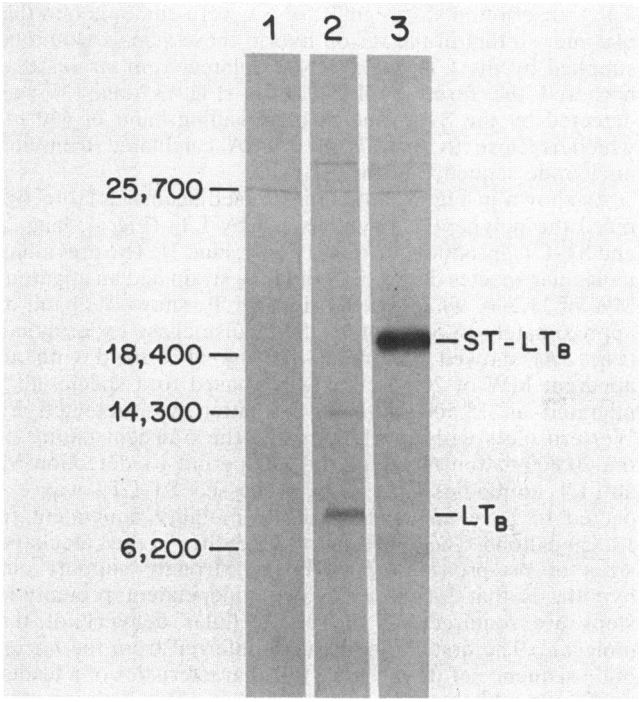

FIG. 4. Protein antibody analysis of peptides synthesized by strain HB101 harboring plasmids pGK22 (ST only, lane 1), pGK40 (LT_B only, lane 2) and pGSK51 (fusion ST-LT_B, lane 3). Sonic extracts of the above strains were fractionated in a 0.1% SDS–20% polyacrylamide separating gel. The proteins were electrotransferred to nitrocellulose membranes and incubated with goat anti-LT; bound antibody was detected by incubation with horseradish peroxidase-conjugated rabbit anti-goat IgG, and the sensitivity was amplified by subsequent incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG. MW markers were bovine trypsin inhibitor (6,200), lysozyme (14,300), β-lactoglobuline (18,400), and α-chymotrypsinogen (25,700).

LT_B-producing strain (Fig. 4, lane 2), a distinct band was detected by the anti-LT_B antibodies. Similar analysis of sonic extracts of strain HB101(pGSK51) (Fig. 4, lane 3), which harbors the fused ST-LT_B gene, allowed the detection of an anti-LT_B-reacting band with a calculated MW of approximately 18,500. The expected MW of the fused ST-LT_B peptide including the complete coding region was 19,705. However, these experimental conditions did not resolve whether the 18,500-dalton band might result from the cleavage of the leader peptide (first 19 amino acids) that should yield a species with a theoretical MW of 17,419.

Detection of ST and LT_B in the fused peptide. To establish whether strains harboring pGSK51 (ST-LT_B) produced both ST and LT_B, the bacterial cultures indicated in Table 1 were grown overnight in T broth at 29 and 37°C; the cells were centrifuged, the cellular density of each culture was adjusted with PBS to 10 mg (wet weight) of cells per ml, and the bacterial suspensions were submerged in an NaCl-ice-water bath and sonicated by 20-s pulses with 40-s intervals. The sonic extracts were used to determine ST activity and LT_B reactivity with antibodies. Strains HB101(pGK22) and HB101(pGK26), both ST-producing strains, had ST activity but not LT_B reactivity (Table 1). Similarly, in the extracts derived from the LT_B-producing strains HB101(pGK40) and HB101(EWD299), LT_B reactivity was detected but no ST activity was found. Sonic extracts of strain HB101(pGSK51) showed both ST activity and LT_B positive on ELISA.

To demonstrate further that the same peptide was responsible for the ST activity and LT_B reaction on ELISA shown in Table 1, sonic extracts of strains HB101(pGSK51), HB101(pGK22), and HB101(pGK40) were incubated with rabbit anti-LT coupled to activated agarose beads (Reactigel-6X). After overnight incubation, the suspension was centrifuged, and the supernatant fluid was assayed for both ST and LT_B. When the extract was from the hybrid-producing strain HB101(pGSK51), the activities before adsorption were 1 U for ST and 3.4 μg/ml for LT; after adsorption, the supernatant was negative for both LT_B and ST. Thus, anti-LT_B coupled to agarose beads adsorbed the hybrid ST-LT_B peptide. As a control, a 1:1 mixture of LT [HB101(pGK40)] and ST [HB101(pGK22)] was sonically disrupted and treated under the same conditions as the extract of HB101(pGSK51). The supernatant of the mixture showed no LT reaction on ELISA, while all the ST remained in this fraction. Attempts to elute the hybrid molecule or LT_B alone from the antibody coupled to the agarose beads were not reproducible, presumably due to the variable inactivation of the peptides under the conditions used.

Processing of the fused ST-LT_B polypeptide. The high yield of transcription from the T7 promoter (present in plasmid pT7-3) by the T7 RNA polymerase (encoded by the compatible plasmid pGP1-2) and the controlled expression of the genes set under this system were used to characterize the processing of the fused ST-LT_B polypeptide. Figure 5 shows the fluorogram of [^{35}S]cysteine-labeled polypeptides in a pulse-chase experiment in which cells of the control strain (Fig. 5B) and of the ST-LT_B-producing strain (Fig. 5A) were fractionated by SDS-polyacrylamide gel electrophoresis. The processing of β-lactamase was observed in the control strain [HB101(pGP1-2)(pT7-3)] and in the ST-LT_B strain [HB101(pGP1-2)(pYK153)]; immediately after the pulse, the prevailing species was the 29,000-dalton precursor, which was completely chased after 60 min to the 27,000-dalton processed enzyme. Figure 5A shows in addition a polypeptide, calculated to be 20,800 daltons, detectable after the pulse and its time-dependent chase to a band with an electrophoretic mobility equivalent to 18,500 daltons. It also shows a faint band that migrated as a 16,300-dalton species. When a similar gel was blotted and analyzed for protein-antibody recognition, the ^{35}S-radioactive 18,500-dalton species was the prevailing band detected by the anti-LT_B antibodies (data not shown). Comparison of the processing of β-lactamase by the two strains showed that the presence of ST-LT_B polypeptide slowed down the conversion of

TABLE 1. ST and LT_B activities detected in strains producing ST, LT, LT_B and fusion ST-LT_B

Plasmid	ST activity[a]				LT (μg/ml)[b]			
	29°C		37°C		29°C		37°C	
	Cells	Sup	Cells	Sup	Cells	Sup	Cells	Sup
pGK22	1	64	64	512	ND[c]	ND	ND	ND
pGK26	1	8	128	16	ND	ND	ND	ND
pGSK51	1	1	4	1	10	0.95	0.4	ND
pEWD299	ND	ND	ND	ND	26	0.87	210	14.7
pGK40	ND	ND	ND	ND	63	1.5	125	4.1

[a] ST activity in cells and supernatant (Sup) was determined by the suckling mouse model (9). The values are the reciprocal of the dilution that gave an intestine-to-body weight ratio of ≧0.08 (negative controls gave a ratio of 0.058 ± 0.008).

[b] LT_B was determined by a GM-1 ganglioside-promoted ELISA with goat anti-rabbit LT_B (40).

[c] ND, Undetected under the assay conditions.

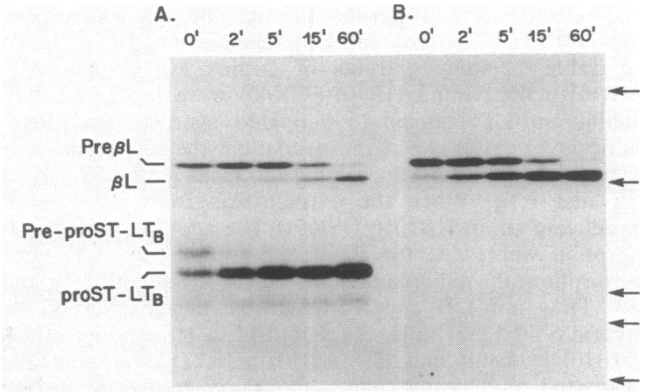

FIG. 5. Fluorogram of SDS-polyacrylamide gel electrophoresis of total cells pulsed for 1 min with [^{35}S]cysteine and chased for the indicated times (in minutes). (A) ST-LT_B-producing strain HB101(pGP1-2)(pYK153) (B) Control strain HB101(pGP1-2)(pT7-3) The M_r-29,000 precursor (proβL) and M_r-27,000 processed β-lactamase (βL) are indicated, as are the precursor (pre-pro-ST-LT_B) and accumulated (pro-ST-LT_B) forms of the fused ST-LT_B polypeptide. The arrows indicate MW markers (top to bottom) bovine trypsin inhibitor (6,200), lysozyme (14,300), β-lactoglobuline (18,400), α-chemotrypsinogen (25,700), and ovalbumin (43,000).

pre-β-lactamase to the mature form. In the control strain (Fig. 5B) 5 min after the chase, the prevailing species was the mature form. However, when the ST-LT_B polypeptide was synthesized and processed by the same strain (Fig. 5A), pre-β-lactamase was the prevailing species even 15 min after the chase. It is also apparent in the fluorogram that the 18,500-dalton ST-LT_B species was detectable as a cell-associated molecule even after a 60-min chase, as was the periplasmic mature β-lactamase (Fig. 5A). The faint 16,300-dalton band also increased with time, but the prevailing species after the chase was the 18,500-dalton molecule.

DISCUSSION

The use of genetic fusions, particularly *lacZ* fusions, has facilitated the detection and study of gene products that are otherwise difficult to analyze and has contributed importantly to the isolation of mutants deficient in the secretion of periplasmic and outer membrane proteins (29, 36). In this study, we fused the 3′ DNA segment that encodes the ST enterotoxin to the 5′ terminus of the LT_B-encoding DNA derived from the LT holotoxin gene. The design of the fusion preserved the inferred amino acid sequence of the ST moiety and added a glycine residue to the first amino acid of the mature LT_B. The resulting DNA had the expected restriction sites (Fig. 2) and nucleotide sequence (Fig. 1, section 5).

We defined the size of the in vivo transcriptional product of the fused genes. When RNA was isolated from unmodified ST-DNA, a 270-nt band was detected by the ST probe (Fig. 3, lane 2). A second band, approximately 490 nt, of lower intensity was also apparent; this band hybridized with a *lacZ* probe (data not shown). Probably this RNA species arises from reading through the ST gene by RNA polymerase initiated at the *lac* promoter of the cloning vector pUC13. The RNA was isolated from exponentially growing cells, and the 270-nt ST mRNA was slightly reduced to a smaller molecular size, suggesting that this mRNA has a long half-life (23). In contrast, RNA isolated from strain HB101(pGK26) showed a wider band detected by the ST probe that spanned from less than 270 to more than 800 nt. The transcription stop signals for ST were eliminated in this plasmid, so that in the fusion hybrid these signals would be supplied by the LT_B gene. RNA isolated from strains that harbored the fused ST-LT_B DNA (Fig. 3, lane 3) was detected by the ST probe as a prevailing band of 630 nt, which is close to the 671-nt mRNA calculated from the nucleotide sequence of the ST gene.

As shown in Fig. 4, antibodies raised against LT_B recognized the polypeptides synthesized by LT- (Fig. 4, lane 2) and ST-LT_B-producing strains (Fig. 4, lane 3). The prevailing molecular species detected in the LT_B strain had an apparent MW of 11,500, while the hybrid ST-LT_B showed a band of approximately 18,500 daltons. The pulse chase experiments (Fig. 5A) showed the initial labeling of a band with an apparent MW of 20,800 that was chased to a species that migrated as 18,500 daltons; the latter was detected by Western blots with anti-LT_B, while the transient nature of the 20,800-dalton molecule did not permit its detection by anti-LT antibodies. The fully processed ST-LT_B was expected to have an electrophoretic mobility equivalent to 13,850 daltons. The 2,300-dalton difference in the calculated sizes of pre-pro- and pro-ST-LT_B strongly support our hypothesis that for ST (13), two independent proteolytic steps are required for the extracellular delivery of the molecule. The first 19 amino acids inferred from the nucleotide sequence of the ST have the characteristics of a leader peptide; it is likely that a signal peptidase (31, 42) cleaves the pre-pro-ST at this point, allowing the protoxin to translocate through the inner membrane to the periplasm. Subsequent proteolysis of the pro-ST at the methionine that precedes the amino-terminal asparagine residue is accomplished by a phenomenon similar to the thermoactivation described previously (13). In the case of the fused pre-pro-ST-LT_B, the initial cleavage to yield pro-ST-LT_B took place, but this species was not efficiently processed (Fig. 5A). The theoretically and experimentally estimated sizes for pre-pro-ST-LT_B and pro-ST-LT_B were 20,800 and 19,705 daltons and 18,500 and 17,419 daltons, respectively; the differences of 1,095 and 1,081 daltons could be accounted for by abnormal electrophoretic migration due to the high cysteine content of the ST moiety or by errors in the experimental estimations. Alternatively, the precursor(s) could be metabolically modified to include non-amino acid components absent in the mature forms of ST and LT_B. The theoretical mass of the fully processed ST-LT_B (13,855 daltons) differed from that of the faint band visible in Fig. 5A (16,300 daltons) by 2,450 daltons. Whether it was a degradation product of the accumulated pro-ST-LT_B or its processing is currently under investigation.

The reduction in the rate of processing of pre-β-lactamase by the synthesis and processing of pre-pro-ST-LT_B seen in the same figure could result from the accumulation in the inner membrane of the pro-ST-LT_B, affecting the binding of pre-β-lactamase to make it accessible to the signal peptidase.

The fused peptide composed of the entire amino-terminal sequence of the extracellular ST and the mature form of the periplasmic LT_B should undergo the initial processing steps of the ST enterotoxin. Nevertheless, the fused peptide could conceivably be processed differently from the natural ST. Thus, the precursor-product relationship detected in the fused ST-LT_B remains to be defined with ST alone.

While natural ST is an extracellular toxin (13, 24) (Table 1), the localization of the toxin encoded by plasmid pGK26 depended on the growth temperature of the host strain; at 29°C it was mainly extracellular and at 37°C it was principally intracellular. The inferred translational product of

pGK26 has three additional amino acids (Gly, Ala, and Leu) at the carboxy terminus of the peptide. Similarly, the fused toxin encoded by pGKS51 remained associated with the cells (Table 1). The common feature of both toxins was that both blocked the natural carboxy terminus of ST. This observation suggests that this terminus is important for the extracellular delivery of ST.

Strains that harbored the fused DNA synthesized both ST and LT_B, as judged by the suckling mouse assay for ST and the GM-1-promoted ELISA for LT_B (Table 1). The level of ST and LT_B was lower than the activity obtained from strains that encoded the two moieties separately. The decrease in activity of the hybrid could arise as a consequence of the topological localization of the hybrid due to the conformation of the ST-LT_B. Whereas LT_B is localized in the periplasm (41) and ST is extracellular (17), the majority of the hybrid ST-LT_B remained cell associated (L.-M. Guzman-Verduzco, E. Powell, and Y. M. Kupersztoch, manuscript in preparation). The quantification of LT_B by the GM-1 ganglioside-promoted ELISA of sonic extracts of ST-LT_B-producing strains was consistently lower than the expected values inferred from the intensity of the band detected by the anti-LT_B antibodies of SDS-lysed cells in Western blots. Although the cross-reacting material was not measured, the difference compared with LT_B alone was very apparent. It is likely that treatment with detergent liberates the hybrid from the membrane, while sonic extraction leaves the molecule associated with the membrane. Thus, it is possible that the activity of the molecule is reduced because it is partially embedded in the membrane. It is also conceivable that the interaction of the two moieties of the fused peptide results in a molecule with different ST and LT activity than that shown by the two individual components; i.e., it is unknown whether the hybrid forms oligomers as LT_B does and whether their reactivity with GM-1 and anti-LT_B antibodies is altered by their structure. It is also unknown whether the presence of the 34 amino acids in pro-ST-LT_B affect the activity of ST and the ganglioside GM-1-binding properties of LT_B.

Klipstein et al. (24) recently immunized volunteers with a synthetic peptide vaccine that consisted of 26 amino acids of an immunodeterminant region of LT_B joined to the 18 amino acids of ST and showed significantly greater titers of antitoxin to both ST and LT_B subunits in the immunized than in the control group. While this approach has the advantage of using a completely defined vaccine, it requires chemical synthesis of the peptides. In contrast, genetically engineered bacteria could deliver the same immunogenic determinants plus determinants involved in other enteric diseases. We are currently exploring the immunogenic properties of the hybrid ST-LT_B.

ACKNOWLEDGMENTS

We thank Eric Hansen, Michael Norgard, and Justin Radolf for their advice on implementation of the Western blot and immunoadsorption experiments, Heather Stieglitz and Robert Munford for the critical review of the manuscript, and Cindy Baselski for typing it. We also thank Frances E. Powell for technical assistance.

The work was supported in part by Public Health Service grants AI-21698 and P01-HD22766 from the National Institutes of Health.

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