Expression of the Cloned Gene for Enterotoxin STb of *Escherichia coli*

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This study involved the construction of hybrid plasmids to produce heat-stable enterotoxin type II of *Escherichia coli* (STb). The translation of the open reading frame for the STb gene *estA* was demonstrated in several ways. Studies using in vivo labeling with [^{35}S]cysteine demonstrated a radiolabeled protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the expected molecular weight of 5,000 for toxin STb. Insertion of translational or transcriptional termination signals into the *Bgl*II site of the *estA* gene blocked the expression of *estA*. The *estA* gene was cloned into high-expression vector pKC30 downstream from the strong p_L promoter. Northern (RNA) blotting assays revealed a 10- to 20-fold increase in mRNA produced by strain C600F(pKC30STb) over other STb-producing strains, compared with little or no increase in enterotoxin activity demonstrated by bioassay. The *estA* gene, with its own promoter and Shine-Delgarno region and a portion of the sequence for the signal peptide deleted, was also inserted under the control of the *tac* promoter. Even after induction of the *tac* promoter by addition of isopropyl-β-D-thiogalactopyranoside, no biologic enterotoxin activity could be identified. Neutralizing antibodies to STb were produced in rabbits by using either a purified OmpF–STb–β-galactosidase fusion protein or a 19-amino-acid synthetic STb peptide coupled to keyhole limpet hemocyanin.

Enterotoxigenic *Escherichia coli* strains produce several enterotoxins, heat-labile types I and II (LT-I and LT-II), heat-stable type I (ST-I or STa), and heat-stable type II (ST-II or STb). These toxins produce diarrhea in animals and humans by increasing intestinal secretion (1, 33, 37). LT-I, LT-II, and STa have been purified, analyzed, and characterized, and their modes of action have been shown to involve stimulation of adenylate cyclase by LT-I and LT-II and guanylate cyclase by STa (11, 26). The mode of action of STb remains unknown, although it has been described as being different from those of LT and STa (12). The fluid secretion caused by STb appears to be mediated by a mechanism different from the alterations in chloride ion secretion or sodium chloride absorption which occur with STa or LT (6, 40).

The proposed gene for STb (*estA*) has been cloned and sequenced (15, 28). On the basis of the nucleotide sequence, *estA* was postulated to code for a protein containing 71 amino acids, including a signal peptide of 23 amino acids and a 48-amino-acid-long mature toxin.

Purification and further studies of STb have been hampered by two problems. (i) Only limited amounts of toxin are produced by strains with the naturally occurring plasmids that contain *estA* (pCG86 and p307) or by a variety of constructed plasmids that contain *estA*. (ii) The only assay for determining STb levels is a cumbersome pig intestinal loop bioassay that measures toxin activity. In this report, we describe the construction of various plasmids and experiments performed to try to overcome these problems.

MATERIALS AND METHODS

Bacterial strains and plasmids. The gene for enterotoxin STb, *estA*, was obtained from plasmids pCHL6 and pUC9STb,

provided by C. H. Lee (15). All of the plasmids used are described in Table 1. The host strains were *E. coli* MH3000 and TK1046 for pORF1 derivatives (41). C600F, N99CI+, and N5151 were used as hosts for pKC30 and its derivatives (2, 31). *E. coli* JM103 was used as the host for pDR540 and its subclones (42). *E. coli* C strain 3456 was used as the host for pCHL6. P3, a wild-type strain of *E. coli* containing the gene for enterotoxin STb, *estA*, and 10405, a wild-type *E. coli* strain without *estA*, were used as controls in the bioassays. All of the strains used are listed in Table 2.

Media. Bacteria were grown in tryptone yeast extract medium or minimal medium A supplemented with 0.25% glucose or 0.20% lactose. Clones were screened for lactose fermentation on MacConkey lactose plates or on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside plates with or without isopropyl-β-D-thiogalactopyranoside (IPTG). In vivo radiolabeling was performed in modified minimal medium A (0.004 M dipotassium phosphate, 0.0022 M potassium biphosphate, 0.0017 M trisodium citrate, 0.0076 M ammonium sulfate, 0.008 M magnesium sulfate, 0.01% yeast extract, 5% glucose, required growth factors). Ampicillin was used at 50 μg/ml, and tetracycline was used at 20 μg/ml.

Materials. Enzymes were purchased from New England BioLabs, Inc., or Boehringer Mannheim Biochemicals and used as recommended by the suppliers. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside, IPTG, and *p*-aminophenyl-β-D-thiogalactosidase were purchased from Sigma Chemical Co. *o*-Nitrophenyl-β-D-galactopyranoside was supplied by Boehringer Mannheim Biochemicals. Oligonucleotides were synthesized by B. Goldschmidt (Department of Biochemistry, New York University School of Medicine). Isotopes were obtained from Dupont, NEN Research Products.

DNA manipulations. Supercoiled plasmid DNA was isolated by Brij 58 lysis and cesium chloride gradient centrifugation as described previously (21). Plasmid DNA for re-

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TABLE 1. Descriptions of plasmids used

Plasmid(s)	Phenotype[a]	Description	Source or reference
pBR322	Ap Tc	Cloning vehicle	4
pCHL6	Ap STb	pBR322 recombinant carrying *estA*	15
pCHL6Ω	Ap Sm Sp	pCHL6 derivative with Ω fragment from pHP45Ω inserted in *estA*	This work
pDB102	Ap β-Gal	pORF2 derivative carrying *tac* promoter and *argR-lacZ* fusion	17
pDR540	Ap	Expression vector with *tac* promoter and replication genes of pBR322	32
pHP45Ω	Ap Sm Sp	pBR322 derivative with the inserted Ω fragment (2.0 kb) consisting of short inverted repeats carrying transcription and translation termination signals and synthetic polylinkers	29
pKC30	Ap	Expression vector with p_L promoter from phage λ and replication genes of pBR322	31
pKC30STb	Ap STb	pKC30 derivative carrying 406-bp *Hind*III-*Bam*HI *estA* fragment from pUC9STb	This work
pKC30STb(TAA)	Ap	pKC30STb recombinant with termination signal inserted in *estA*	This work
pORF1	Ap	Open reading frame expression vector for *lacZ* fusion proteins	41
pPH1	Ap	pORF1 derivative with *ompF-lacZ* fusion	This work
pPH14	Ap	pORF1 derivative with *ompF-estA-lacZ* fusion	This work
pR3-76	Ap β-Gal	pDB102 derivatives with a fragment of *estA* fused to *lacZ*	This work
pRL3 and pRL65	Ap	pCHL6 recombinants with *tac-estA* fragments from pR3 and pR65 inserted to form the complete STb structural gene	This work
pUC9STb	Ap	pUC9 derivative with *estA* lacking the −35 promoter region	15

[a] Ap, Ampicillin resistance; Tc, tetracycline resistance; Sm, streptomycin resistance; Sp, spectinomycin resistance; STb, enterotoxin STb activity; β-Gal, β-galactosidase activity.

striction analysis of clones was isolated by the method of Holmes and Quigley (10). Restricted fragments were separated on and subsequently isolated from low-melting-point agarose gels (SeaPlaque; FMC Corp., Marine Colloids Div.). Dideoxynucleotide sequence analysis was performed by either cloning the appropriate fragments in M13mp19 (24, 35) or using double-stranded DNA and Sequenase from United States Biochemical Corp. (38). DNA probes were labeled by either nick translation (30) or 5' end labeling with T4 polynucleotide kinase (20). Transformation of *E. coli* strains was performed by the method of Kushner (13).

Colony hybridization. Colony hybridization was performed as described by Maas (18). When oligonucleotides were used as probes, hybridization was performed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 23°C, and the stringent wash was performed in 6× SSC at successively increasing temperatures (23, 30, 38, and 45°C) (42).

In vivo radiolabeling. In vivo radiolabeling was performed by growing cells in 2 ml of modified minimal medium A with 1 μmol of [^{35}S]cysteine (specific activity, 12 mCi/mmol) for 24 h at 37°C. The culture supernatant was analyzed by sodium dodecyl sulfate–15% polyacrylamide gel electropho-

TABLE 2. *E. coli* strains used

Name	Relevant characteristics	Source (reference)
MH3000	*leu lac ompR101* Str^r	T. J. Silhavy (41)
TK1046	*lac ompBcs1* Str^r	T. J. Silhavy (41)
C600	*thr leu thi lac*	B. J. Bachmann (2)
N99CI+	Wild-type λ lysogen	A. Shatzman (31)
N5151	Heat-inducible λ lysogen mutant derived from SA500	A. Shatzman (31)
JM103	Δ(*lac-proAB*) *thi* Str^r F'(*traD36 proAB lac*Iq ZΔM15)	Pharmacia (42)
3456	*met lac thi*; derivative of *E. coli* C	W. Goebel
10405	*estA*; porcine isolate of wild-type *E. coli*	C. L. Gyles (36)
P3	Porcine isolate of wild-type *E. coli*[a]	C. L. Gyles (36)

[a] Enterotoxin STb-producing strain.

resis (SDS-PAGE) (14). The gel was silver stained (23), soaked in 10% glycerol to prevent cracking, and dried under a vacuum on a backing of 3 MM filter paper. Kodak XAR-5 X-ray film was used for autoradiography with one Dupont Cronex Lightning-Plus intensifying screen.

Bioassay for enterotoxin STb activity. Bioassays for STb enterotoxic activity were performed by both C. Weikel and C. Gyles in ligated intestinal loops of 4- to 6-week-old weaned piglets as previously described (5, 36). Neutralization of enterotoxin STb activity was determined by combining antisera to STb with the culture supernatant before injecting the supernatant into the intestinal loops. The volume/length ratios were determined and compared with those of controls, which included supernatant combined with preimmune sera and supernatant, antisera, or media alone.

Purification of fusion protein. The OmpF–STb–β-galactosidase tribrid protein was purified from a culture of strain TK1046(pPH14). Cells were harvested, washed twice in 50 mM sodium phosphate buffer (pH 7), and lysed by sonic oscillation at 5 to 10°C. The lysate was centrifuged at 15,000 × *g* for 30 min. The supernatant was subjected to 40% ammonium sulfate precipitation for 1 h and centrifuged at 10,000 × *g* for 30 min. The ammonium sulfate precipitate was suspended in Arg buffer (20 mM Tris hydrochloride, 10 mM magnesium chloride, 10 mM β-mercaptoethanol [pH 7.5]) and dialyzed against this buffer. The dialysate was brought to a final concentration of 1.6 M sodium chloride, purified by affinity chromatography by using a *p*-aminophenyl-β-D-thiogalactosidase CH-Sepharose 4B column as described by Ullmann (39). Fractions were collected and analyzed for β-galactosidase activity as previously described (25). β-Galactosidase-rich fractions were pooled and dialyzed against Arg buffer without sodium chloride. Final purification of the fusion protein was achieved by anion-exchange chromatography using a Mono Q column attached to a fast-protein liquid chromatography system (Pharmacia). The column was developed by a 0 to 1 M NaCl gradient. Fractions showing high β-galactosidase activity and exhibiting a single band in SDS-PAGE were pooled, dialyzed against Arg buffer, and concentrated in an Amicon Centricon YM-10 microconcentration unit.

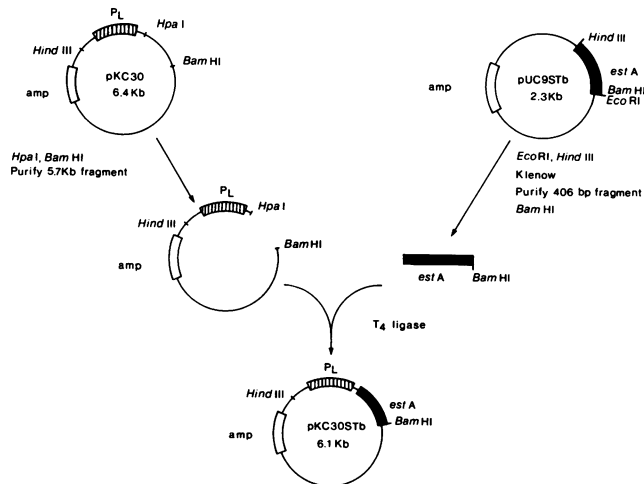**Antibody production.** Antibodies to the OmpF–STb–β-

FIG. 1. Construction of pKC30STb. bp, Base pair; Kb, kilobase.

galactosidase tribrid protein and the synthetic STb peptide coupled to keyhole limpet hemocyanin (KLH) were elicited in rabbits by a previously described protocol (27). The immunoglobulin fraction was prepared from immune sera as described by Harboe and Inglid (9). Antisera to the OmpF–STb–β-galactosidase tribid protein and the STb-KLH-coupled protein were adsorbed against β-galactosidose–Sepharose 4B and KLH-Sepharose 4B affinity beads, respectively. One-milliliter samples of whole sera were adsorbed with 1 ml of the affinity beads, each coupled with 10 mg of the appropriate protein.

Synthetic STb peptide. A synthetic STb peptide was constructed for use as a marker on SDS-PAGE and for antibody production. A 19-amino-acid segment of STb, containing six of the nine charged amino acids in the STb protein to allow enhanced antibody production, was synthesized by D. Schlesinger (22; Department of Cell Biology, New York University) (see Fig. 3). The synthetic peptide was coupled to KLH by a glutaraldehyde coupling procedure and subsequently used in antibody production.

Isolation of total RNA. A modification of a guanidinium isothiocyanate-hot phenol procedure was used (7). A 2-ml volume of a bacterial culture was grown to an A_{580} (Lumetron colorimeter) of 0.3. Bacterial cells were harvested, and pellets were washed with RNA buffer (8). Cells were lysed by addition of 4 ml of 4 M guanidinium isothiocyanate, and the temperature was increased to 60°C. Water-saturated phenol preheated to 60°C was added to the lysis mixture. Four milliliters of chloroform-isoamyl alcohol (24:1) was added, and the lysate was left at 60°C for 60 min. Samples were cooled on ice and centrifuged at 4,000 × g for 10 min at 4°C. The aqueous phase was re-extracted with phenol-chloroform as described above. Ether extraction was performed three times to remove the chloroform and residual phenol. The aqueous phase was mixed 1:1 with 4 M lithium chloride (43), kept on ice for 2 to 3 h, and centrifuged at 10,000 × g for 10 min. The pellet was suspended in 5 mM EDTA, vortexed, boiled for 1 min, and centrifuged again at 10,000 × g for 10 min to remove undissolved material. The nucleic acids in the supernatant were precipitated twice with ethanol. The RNA was recovered by centrifugation and stored at a concentration of 10 μg/ml in sterile distilled water. The amount of RNA was measured by spectrophotometric reading, and an A_{260} of 1.0 corresponded to approximately 40 μg/ml for single-stranded RNA. Samples were run
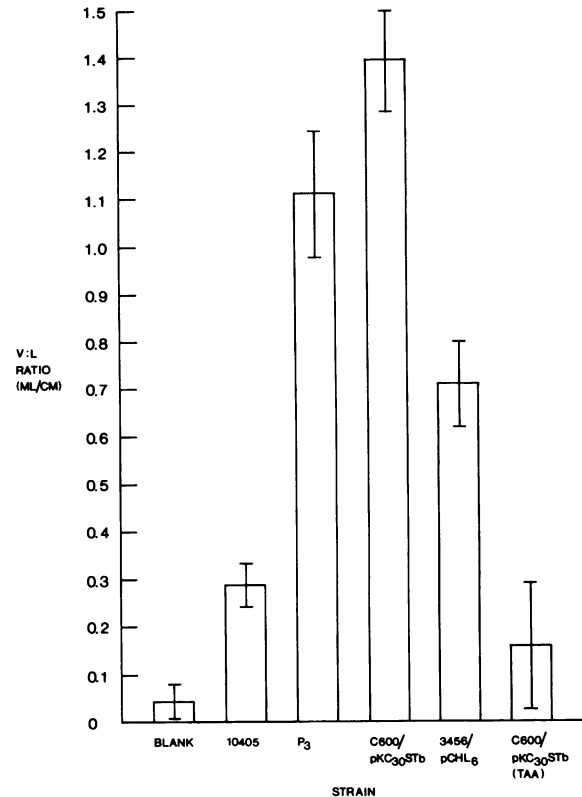

FIG. 2. Bioassay results. Volume-length (V:L) ratios are expressed as means of 10 or more measurements {except for 3456 (pCHL6) (n = 8) and C600[pKC30STb(TAA)] (n = 5)} ± the standard errors. Two-tailed paired t tests showed that the mean volume-length ratios for P3, C600(pKC30STb), and 3456(pCHL6) were all statistically significantly greater than the mean volume-length ratios for the blank or 10405 (P < 0.01). There was no statistically significant difference between the means for P3 and C600(pKC30STb) or among the means for the blank, 10405, and C600[pKC30STb(TAA)]. The blank was an intestinal loop without a culture supernatant instilled into it.

on a 1.5% agarose–2.2 M formaldehyde gel (16), transferred by capillary blotting to nitrocellulose, and hybridized by using a nick-translated STb probe.

RESULTS

Hybrid plasmid construction. Several plasmids were constructed either to place the *estA* gene under the control of a strong promoter to create STb-hyperproducing strains or to create *estA-lacZ* fusions which would produce STb–β-galactosidase fusion proteins for use in antibody production. Figure 1 shows the construction of pKC30STb. The *estA* gene, containing the endogenous −10 consensus sequence, ribosome-binding site, complete signal peptide, and complete protein sequence of STb, was inserted 174 base pairs downstream from the translational start site for the N protein of bacteriophage λ and 341 base pairs downstream from the transcriptional start site of the p_L promoter of phage λ (34). The presence of the *estA* insert was confirmed by colony hybridization and restriction analysis. SDS-PAGE of the 100-fold-concentrated supernatant of strain C600(pKC30STb) demonstrated an appropriate-molecular-weight band of approximately 5,000 for STb. Bioassay testing of supernatants of the same strain consistently demonstrated enterotoxin
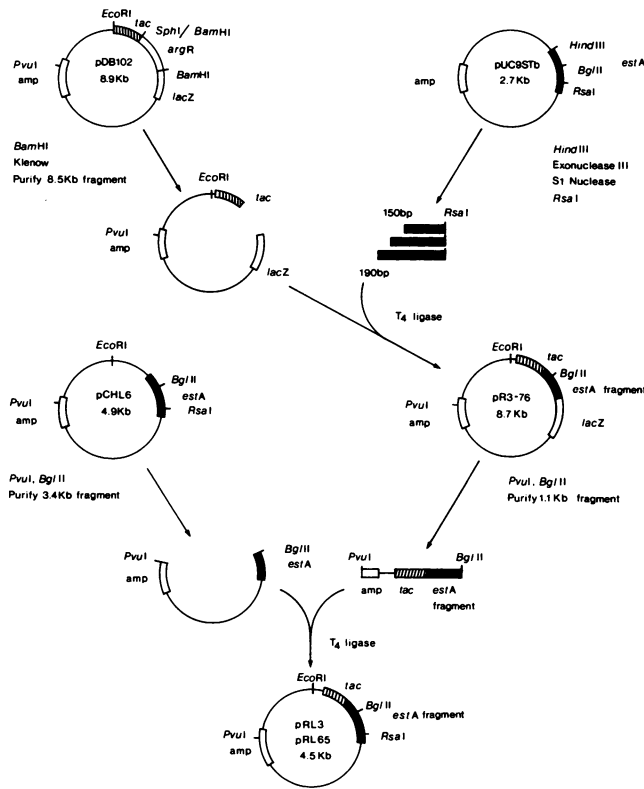
FIG. 3. Construction of *tac-estA* fragment-*lacZ* fusions and *tac-estA* hybrid plasmids. Fragments (150 to 190 base pairs [bp]) of *estA* from pUC9STb were separated on a 5% acrylamide gel and electro-eluted into a dialysis bag before ligation (19). pR3-76 is representative of the five clones with the promoter regions and portions of the sequence for the signal peptide of *estA* removed (Fig. 4). The 1.1-kilobase (Kb) *Pvu*I-*Bgl*II fragments from pR3 and pR65 were inserted into pCHL6 to reconstruct the complete sequence of the mature enterotoxin STb.
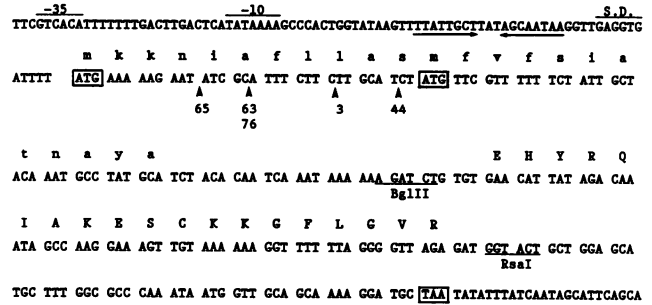

FIG. 4. *tac-estA-lacZ* fusion clones. The arrowheads indicate deletion endpoints in *estA* confirmed by dideoxy sequencing of the appropriate M13mp19 clones. The numbers identify the clones tested (Table 3). The deletion endpoints all occur in the region that codes for the signal peptide (indicated by lowercase single-letter symbols for amino acids) before a second ATG start codon. The −35, −10, and Shine-Dalgarno (S.D.) sequences are indicated. Initiation and termination sites are boxed. The 19-amino-acid synthetic peptide of STb used as a control in SDS-PAGE and in antibody production is indicated by uppercase single-letter symbols for amino acids.

activity equal to that of wild-type STb-producing strain P3 (Fig. 2).

A second attempt to create an STb-hyperproducing clone entailed deletion of the upstream native control region of *estA* and inserting the *estA* gene under the control of the *tac* promoter. Furthermore, this construct fused *estA* to *lacZ* to score clones by measuring β-galactosidase activity for presumptive concomitant STb hyperproduction (Fig. 3). Hybrid plasmids (pR3-76) were obtained by selecting JM103 transformants for ampicillin resistance and scoring them for β-galactosidase activity on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside plates. Over 700 transformants were tested for the presence of the *estA* fragment by colony hybridization. Of those, 190 hybridization-positive transformants were tested quantitatively for β-galactosidase activity (25). Eight transformants with the highest β-galactosidase activities were selected to sequence the *tac-estA* region and determine what portion of the *estA* gene had been deleted. M13mp19 clones for sequencing were obtained for only five of the eight transformants. All of the selected transformants demonstrated deletion of the native upstream control region of the *estA* gene and variable portions of the signal peptide region (Fig. 4). All of these clones had high levels of β-galactosidase activity, although less than that of parent plasmid pDB102 (Table 3). IPTG induction of the above-

described transformants resulted in increased production of the STb–β-galactosidase fusion protein, as demonstrated by SDS-PAGE of the cell contents, followed by silver staining (data not shown). Two clones were selected for deletion of the *lacZ* region and reconstruction of the complete sequence for the mature STb enterotoxin with the partially deleted signal peptide region under the control of the *tac* promoter (Fig. 3). The derived plasmids, designated pRL3 and pRL65, were transformed into JM103. Sequence analysis confirmed the presence and proper orientation of the *tac* promoter, the partially deleted signal peptide sequence, and the complete sequence for the mature STb protein. Analysis of unconcentrated and concentrated (100-fold) samples of supernatant, cytoplasm, and cell membrane fractions failed to demonstrate an appropriate-molecular-weight band on SDS-PAGE gels or biological enterotoxin activity for strain JM103(pRL3 or pRL65).

Insertional mutagenesis. To confirm *estA* as the structural gene for enterotoxin STb, transcriptional and/or translational termination signals were inserted into the intact *estA* gene (Fig. 5). These clones were then tested by in vivo radiolabeling for loss of the radiolabeled STb band and by bioassay for loss of enterotoxin activity. The SDS-PAGE gel and the autoradiogram of that gel (Fig. 6) demonstrated the presence of a radiolabeled band of the appropriate molecular weight for enterotoxin STb only in the strain with the intact *estA* gene. There was a second radiolabeled band with an

TABLE 3. β-Galactosidase activities of *estA-lacZ* fusion clones

Clone no.[a] or strain	Mean ± SD β-galactosidase activity[b] (U/min per ml)
3	1,149.2 ± 694.4
44	1,308.3 ± 565.7
63	425.6 ± 228.2
65	416.8 ± 185.1
76	482.2 ± 324.6
pDB102	1,401.8 ± 717.4
JM103	2.8 ± 4.2

[a] The numbers identify the clones with the deletion endpoints in *estA* shown in Fig. 4.

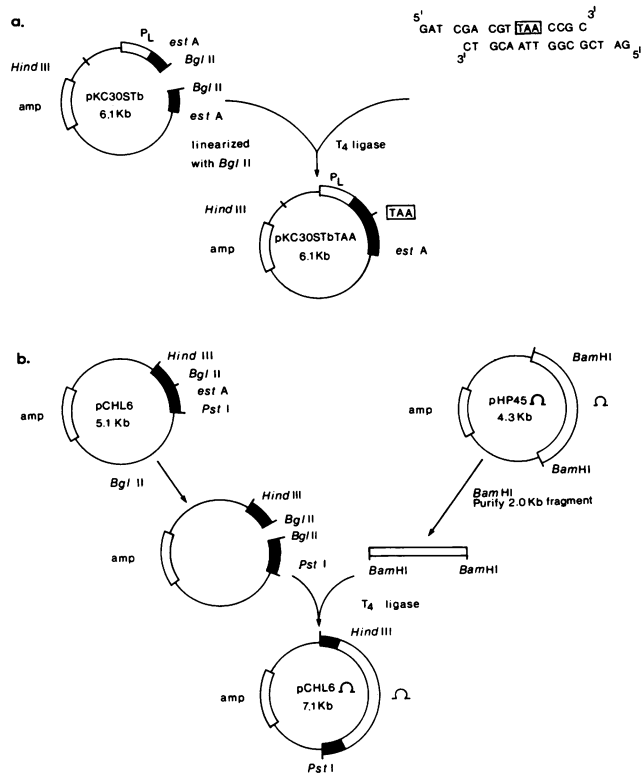[b] The values reported are based on at least five separate measurements.

FIG. 5. Insertional mutagenesis of the *estA* gene. (a) The renatured oligonucleotide containing the TAA translation termination signal was inserted in frame with *estA*. (b) An Ω fragment with transcriptional and translational termination signals was inserted into the *Bgl*II site of *estA*. Kb, Kilobase.

approximate molecular weight of 40,000 which was not identified. Bioassay results confirmed that STb activity was present only in strains with an intact *estA* gene and absent wherever an insertion was made. Bioassay results for strain C600[pKC30STb(TAA)] are shown in Fig. 2.

Fusion protein construction. The cloning strategy for construction of hybrid plasmid pPH14, which is capable of producing an OmpF–STb fragment–β-galactosidase fusion protein, is shown in Fig. 7. Plasmid pPH1 (Fig. 7) served as a control. Hybrid plasmids pPH1 and pPH4 were transformed into MH3000 and TK1046. Transformants were selected for ampicillin resistance and scored for β-galactosidase activity on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside indicator plates. Measurable β-galactosidase activity, colony hybridization for the *estA* fragment, and restriction analysis confirmed the correct orientation for in-frame reading of the fused genes.

Antisera to STb. Strain TK1046(pPH14) was used to purify the fusion protein (OmpF–STb fragment–β-galactosidase), and this purified protein was used to raise antibodies in rabbits. Gel immunodiffusion demonstrated precipitin bands between the undiluted and 1:2 dilution preparations of antiserum and the fusion protein. Immunoblotting with undiluted anti-STb–β-galactosidase serum showed cross-reactivity with non-STb-producing strains. Testing of the antiserum to the fusion protein in the bioassay demonstrated blocking of enterotoxin activity of a standard STb-producing strain, whereas preimmune serum and anti-β-galactosidase serum showed no neutralization of the secretory action of the same STb-producing strain (data not shown).
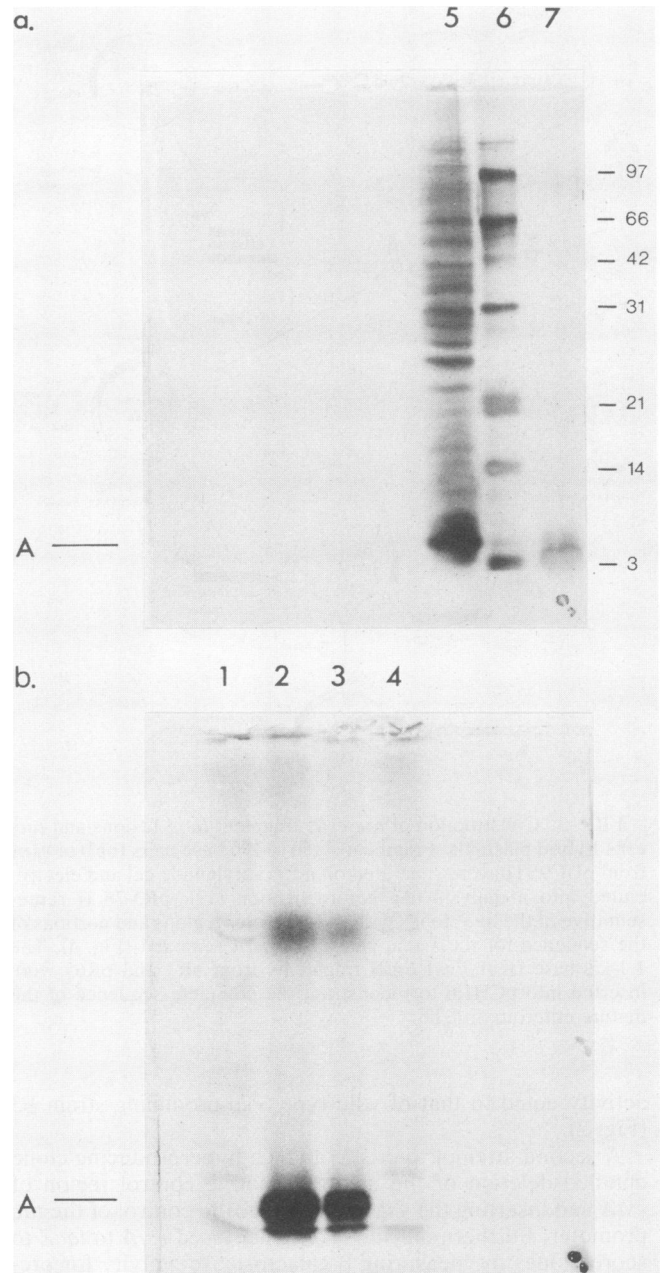

FIG. 6. In vivo radiolabeling of STb. (a) Silver-stained, dried SDS-PAGE gel. Lanes; 5, supernatant of C600(pKC30STb) concentrated 100-fold; 6, Bio-Rad low-molecular-weight protein standard with insulin added (molecular weights: 97,400, 66,200, 42,699, 31,000, 21,500, 14,400, and 3,000); 7, STb synthetic peptide (15 μg). (b) Autoradiogram of the gel in panel a. The samples were the unconcentrated supernatant fraction of the following in vivo-radiolabeled cultures. Lanes: 1, C600(pKC30) (20 μl); 2 and 3, C600 (pKC30STb) in duplicate (20 and 10 μl, respectively); 4, C600 [pKC30STb(TAA)] (20 μl). The position of toxin STb band A is shown.

To obtain a more specific antiserum to STb, a 19-amino-acid peptide which contained six of the nine charged amino acids in toxin STb was synthesized (Fig. 4). This synthetic STb peptide did not demonstrate enterotoxin activity on bioassay. This peptide was then coupled to KLH and used as an immunogen. Subsequent testing by Ouchterlony gel
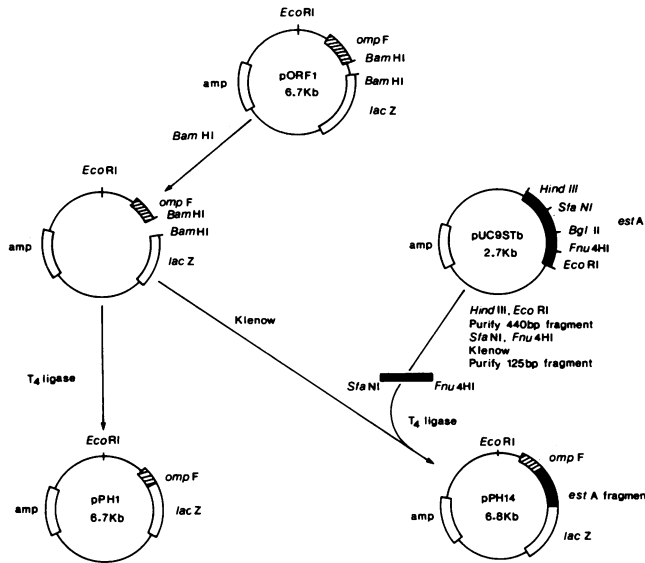
FIG. 7. Construction of an *ompF-estA-lacZ* fusion and *ompF-lacZ* fusion in pORF1. Kb, Kilobase; bp, base pair.

immunodiffusion showed precipitin bands between supernatant preparations of C600(pKC30STb) that were concentrated 100-fold and STb-KLH antiserum that was undiluted or diluted 1:2. Immunoblotting with undiluted STb-KLH antiserum as the primary antibody and alkaline phosphatase–goat anti-rabbit immunoglobulin G-coupled antibody as the second antibody demonstrated detection of 150- to 300-ng quantities of the synthetic STb peptide but also showed cross-reactivity with supernatant and cytoplasm fractions of non-STb-producing strain JM103(pDB102). Similarly, Western blots (immunoblots) of 20% native polyacrylamide gels (Phastgel; Pharmacia) showed immunostaining of a low-molecular-weight band and a high-molecular-weight (approximately 65,000) band in 100-fold-concentrated supernatants of C600(pKC30STb) and JM103(pDB102). In the pig loop bioassay, anti-STb-KLH sera demonstrated enterotoxin-neutralizing activity against C600(pKC30STb). Eight milliliters of the culture supernatant of C600(pKC30STb) was incubated for 30 min with 0.5 ml of culture medium, preimmune serum, or anti-STb–KLH serum before injection into a pig loop intestine. The means of three volume-length ratio measurements for samples incubated with culture medium or preimmune serum were 3.20 and 3.13 ml/cm, respectively. The mean volume-length ratio for samples incubated with anti-STb–KLH serum was 0 ($n = 4$).

Northern (RNA) blotting. Northern blots of total RNA from several strains were analyzed by hybridization with a ^{32}P-labeled *estA*-containing probe. The following strains made no probe-specific RNA: JM103, C600F, C600F (pDB102), and C600F(pUC9STb). RNA samples from C600F(pKC30STb) showed a hybridization band significantly stronger than that from 3456(pCHL6) or JM103(pRL3 or pRL65). Serial dilutions of RNA samples from C600 (pKC30STb) showed that the hybridization reaction was approximately 10 to 20 times as strong as with a sample from 3456(pCH60) or JM103(pRL3 or pRL65). IPTG induction was used whenever appropriate.

DISCUSSION

These data confirm that the open reading frame designated by Lee et al. (15) and Picken et al. (28) represents the structural gene for enterotoxin STb. Cloning of the designated open reading frame, *estA*, into pKC30 resulted in expression and secretion of measurable enterotoxin activity in the pig ileal loop assay. In vivo radiolabeling studies demonstrated that insertion of a translational termination codon into the *Bgl*II site within the open reading frame resulted in loss of a single protein band of the expected molecular weight for enterotoxin STb, as well as loss of biologic enterotoxin activity.

In our studies, STb was identifiable either by bioassay or as a band with a molecular weight of approximately 5,000 on SDS-PAGE gels. Visualization of this band required either in vivo radiolabeling of the protein with [^{35}S]cysteine or concentration of culture supernatants 100-fold in concert with silver staining of the gel. This emphasizes that only very small amounts of STb are produced and/or are necessary for enterotoxin activity. Only crude estimates of the amount of enterotoxin STb produced might be made from dilutional studies of SDS-PAGE gels and bioassays. Ideally, an immunoassay might provide a rapid and more sensitive method for detecting and quantitating STb.

In this study, a purified preparation of fusion protein OmpF–STb–β-galactosidase was immunogenic. The STb component of the fusion protein had two amino acids deleted from the carboxy terminus and four amino acids deleted from the amino end of the mature toxin. This truncated STb molecule, fused to OmpF and β-galactoside, lacked enterotoxin activity but was capable of eliciting neutralizing antibodies to STb. An even further truncated form of the STb toxin, the 19-amino-acid synthetic peptide, also lacked enterotoxin activity but elicited neutralizing antibodies to STb when coupled to KLH. Both anti-OmpF–STb–β-galactosidase and anti-STb–KLH sera reacted weakly at low dilutions with the enterotoxin and contained cross-reacting antibodies to unknown antigens in non-STb-producing strains. Establishment of an immunoassay for STb will require a large purified amount of toxin STb (synthetic or natural) that may have to be coupled to a carrier molecule to produce the large amounts of specific antisera necessary for an immunoassay.

We have tried to create STb-hyperproducing strains by cloning *estA* under the control of the p_L promoter from phage λ and the consensus promoter *tac* (*trp-lac*). Other investigators have created STb-hyperproducing plasmids by deleting the endogenous regulatory sequences of the *estA* gene and inserting it under the control of the T7 phage promoter (R. G. Urban and L. A. Dreyfus, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, B79, p. 43). In strains with inducible T7 RNA polymerase, STb was secreted in large quantities. Strain C600F(pKC30STb) demonstrated an amount of enterotoxin activity similar to those of STb-producing strains P3 and 3456pCHL6, and no enterotoxin activity was demonstrated in the cytoplasmic or periplasmic fraction. However, Northern blotting mRNA analysis of this strain demonstrated approximately a 10- to 20-fold-stronger signal than in other STb-producing strains. Possible explanations for this discrepancy are either limitation of STb expression at a translational level or destruction of toxin STb after synthesis.

The *tac-estA-lacZ* constructs involved deleting the natural *estA* control regions before insertion of the *estA* gene under the control of the *tac* promoter. The selected clones all had deletions of the complete upstream region, including deletions into the region that codes for the charged amino end of the signal peptide. Transcription of fusion protein STb–β-galactosidase or the STb protein with a truncated signal

peptide from plasmid pR3-76 or pRL3 and pRL65 presumably began at the ATG start site located downstream from the deletion sites (Fig. 3). Our procedure, selecting clones which hyperproduced β-galactosidase on induction with IPTG probably selected against clones with an intact signal peptide region because of overproduction lethality. The intact signal protein region allows secretion of the STb–β-galactosidase fusion protein, which leads to cell death when a large amount of fusion protein is produced, as described by Benson et al. for other secretable fusion proteins (3). The constructed clones, pRL3 and pRL65, which contained the *tac* promoter, the partial signal peptide sequences, and the intact mature toxin STb sequence showed no enterotoxin STb activity in the cytoplasm, cell membrane, or supernatant fraction. There was also no evidence of toxin protein in any of these fractions (concentrated 100-fold) by SDS-PAGE. It is not clear why we could not detect the STb protein presumably linked to the residual signal peptide.

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