

High-Level Production of *Escherichia coli* STb Heat-Stable Enterotoxin and Quantification by a Direct Enzyme-Linked Immunosorbent Assay

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Received 25 April 1990/Accepted 7 August 1990

A convenient and sensitive enzyme-linked immunosorbent assay (ELISA) for the STb heat-stable enterotoxin of *Escherichia coli* was developed and used to quantify STb production by strains with a high level of expression. Based on an antigenic profile of the secreted form of STb, a synthetic peptide (STb3-27) spanning the major predicted epitope was synthesized, coupled to keyhole limpet hemocyanin, and used to immunize rabbits. Anti-STb3-27 antibodies were affinity purified on a synthetic peptide-Sepharose 4B column and used in a direct-binding STb ELISA. Based on a highly purified form of toxin as a standard, the ELISA detected as little as 1 to 2 ng of STb from crude culture filtrates. ELISA data revealed that natural STb-producing strains elaborate little STb in defined-medium cultures relative to that elaborated by a recombinant strain harboring a cloned copy of the *estB* gene. Replacement of the endogenous STb promoter with any of several highly active promoters, including a bacteriophage T7 promoter, a β -galactosidase promoter, and a tryptophan- β -galactosidase hybrid (*tac*) promoter, increased the yield of STb 10- to 20-fold over levels obtained by an *E. coli* strain harboring the recombinant *estB* gene. The high level of STb antigen detected by the ELISA correlated with intestinal secretory activity. The combination of a convenient assay and effective hyperproduction of STb will serve as a basis for a large-scale toxin purification strategy.

Enterotoxigenic *Escherichia coli* (EPEC) causes serious diarrheal disease in humans and domestic animals by the production of protein enterotoxins. One class of EPEC toxins, the heat-labile enterotoxins (LTI and LTII), are structurally and functionally related to cholera toxin and appear to mediate secretion by similar mechanisms (4, 5, 11, 14). The heat-stable enterotoxins produced by EPEC, STa and STb, are distinct from the heat-labile enterotoxins in structure, antigenicity, and biological activity. STa and STb share some interesting features, in that the *estA* and *estB* genes are both integral parts of separate bacterial transposons (16, 19, 26, 27), and the encoded products are cysteine-rich (20, 23, 27). STb and STa are often produced by the same EPEC isolate from swine, and STb appears to be a significant contributor to swine colibacillosis, in that the *estB* gene is the most common toxin gene found in association with EPEC of diseased swine (22). Despite its prevalence in swine, the STb gene is uncommon in EPEC of human origin, and STb does not appear to be a cause of human diarrheal disease (7, 33).

Despite similarities in heat stability and association with transposons, STa and STb appear to be totally distinct toxins. STa is an 18- or 19-amino-acid peptide that is devoid of basic residues and contains 6 cysteine residues with no free sulfhydryl groups (6, 29, 31). Export of STa to the surrounding medium by *E. coli* proceeds via a two-step process, the final step of which occurs on the extracellular side of the outer membrane (24). In the gut, STa binds to a unique receptor located in the brush border membrane (9, 10) and activates particulate guanylate cyclase in intestinal

epithelial cells (12). Accumulation of cyclic GMP in the gut mucosa correlates with the resulting secretory response (8). In contrast to STa, relatively little information concerning the biochemistry and mode of STb action is available; however, the gene encoding STb has been cloned and sequenced (20, 23). Furthermore, the export of STb proceeds via a single processing step (18) distinct from that of STa (24). The secreted form of STb is a 48-amino-acid, lysine-rich peptide (18). Therefore, STa and STb are totally distinct toxins with very different apparent biochemical properties. Although the intestinal secretory action of STb is poorly understood, the available data indicate that STb induces gut fluid secretion by a previously uncharacterized mechanism that is apparently independent of changes in cyclic nucleotide levels (17, 32).

Currently, no convenient in vitro assay for STb exists. An enzyme-linked immunosorbent assay (ELISA) for STb has been reported (13); however, the assay depends on the availability of STb-protein A and STb- β -galactosidase gene fusion products as sources of immunogen and screening antigen, respectively. Although sensitive and specific, the requirement for the specialized gene constructs has committed the assay to limited widespread use. Another obstacle in STb research is the poor yield of STb from naturally occurring STb-producing strains, thus complicating STb purification. In this report we address the problems of toxin assay and production. We describe here the development of an STb-specific ELISA that can be conveniently reproduced in other laboratories and the construction of recombinant STb gene plasmids which greatly improve the yields of STb from defined-medium cultures. Our results provide important advances toward defining optimal conditions for toxin production and recovery and provide the basis for a rational

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approach to circumventing the inherent problems associated with STb purification.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* HB101 (2) was used in all experiments to harbor recombinant plasmids. In some experiments, competent *E. coli* DH5 α (BRL Life Technologies, Inc., Gaithersburg, Md.) strains were used as transient plasmid hosts from which recombinant plasmid DNA was isolated and introduced into HB101 by transformation following treatment with CaCl₂ (21). The plasmids pBR322 (1) and pUC18 (35) were obtained from BRL Life Technologies, Inc., and pDR540 (25), which was used as a source of the *trp-lac* (*tac*) promoter (25), was obtained from Pharmacia LKB Biotechnology, Inc., Piscataway, N.J. A bacteriophage T7-promoted STb expression plasmid, pUD1, was constructed in our laboratory and has been described elsewhere (18). We obtained pCHL6 (20), an STb-encoding recombinant derivative of pBR322, from C. H. Lee. The bacteriophage T7 RNA polymerase encoding plasmid, pGP1-2 (30), was obtained from Stan Tabor.

Media, growth conditions, and STb production. LB medium (21) was used for routine maintenance of bacterial strains and for transformation experiments. M9 minimal medium (21) supplemented with 0.1% Casamino Acids (Difco Laboratories, Detroit, Mich.) was used for production of STb from recombinant and natural STb-producing strains. For all strains harboring recombinant plasmids, carbenicillin (100 μ g/ml) was added to the culture medium. Cultures of HB101(pUD1, pGP1-2) contained carbenicillin and kanamycin (50 μ g/ml). When noted, isopropyl- β -D-thiogalactopyranoside (BRL Life Technologies, Inc.) was added to cultures of HB101 harboring pPD11 and pPD21 to induce the *lac*- and *tac*-promoter STb gene cassettes encoded by these two expression plasmids, respectively. Routinely, broth cultures were started from single bacterial colonies and grown at 37°C with vigorous shaking (200 rpm) until the mid-logarithmic phase of growth was attained; portions of these cultures were then used as seeds for overnight cultures in fresh medium. Overnight cultures for STb production were inoculated with seed culture to an optical density at 600 nm (OD₆₀₀) of 0.05. Following approximately 18 h of incubation (as described above), samples of cultures were taken for viable bacterial cell analysis (by dilution plate counting), and the remaining culture was centrifuged at 4,500 \times *g*. The culture supernatant fraction was then filter sterilized through low-protein-binding, 0.45- μ m-pore-size membrane filters (Gelman Sciences, Ann Arbor, Mich.). The level of STb in culture filtrates was determined by the weaned pig intestinal loop assay (34) and by the ELISA described below. Production of STb from the T7 bacteriophage-promoted expression plasmid pUD1 was performed by growing HB101 harboring pUD1 and pGP1-2 (30) at 30°C with aeration until the mid-logarithmic phase of growth (OD₆₀₀, 0.5 to 1.0). The culture was then shifted to 42°C for 20 min and then down to 37°C for 18 h. The bacterial culture was then treated as described above.

Pig intestinal loop assay for STb. The secretory response to STb was measured in weaned pigs by a previously reported ligated intestinal loop assay (34).

Generation of STb-specific affinity-purified rabbit antibody.

(i) **Synthesis of STb3-27 and coupling to KLH.** A synthetic peptide representing residues 3 to 27 of processed STb (18) (codons 26 to 50 of a previously reported sequence [20, 23]) was synthesized at the Synthesis Core Facility Laboratory,

University of Texas Medical Branch, on a Biosearch 9600 peptide synthesizer (MilliGen/Biosearch, Novato, Calif.). For immunogen, synthetic peptide was coupled to keyhole limpet hemocyanin (KLH; Calbiochem Corp., La Jolla, Calif.) by using maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS; Pierce Chemical Co., Rockford, Ill.) as described below. To 5 mg of KLH dissolved in 0.25 ml of phosphate buffer (0.05 M; pH 7.5) was added 0.4 mg of MBS (50 \times molar excess of MBS, assuming an average molecular weight of 200,000 for KLH) dissolved in 0.25 ml of dimethyl sulfoxide. The reaction was allowed to proceed for 30 min at room temperature. Unreacted MBS was removed by Sephadex G-50 (Pharmacia LKB Biotechnology, Inc.) gel filtration chromatography. A 50 \times molar excess of synthetic peptide (3.6 mg), which was dissolved in 0.1 ml of phosphate-buffered saline (PBS), was added to the MBS-activated KLH. In addition, 2 \times 10⁶ cpm of ¹²⁵I-labeled peptide (described below) was added as an indicator of coupling efficiency. Peptide and MBS-activated KLH were mixed at room temperature for 2 h and then dialyzed overnight against 0.05 M phosphate buffer. A portion of the dialyzed preparation was assayed for peptide by liquid scintillation counting. Under the conditions described above, greater than 70% of the added peptide was routinely coupled to KLH.

(ii) **¹²⁵I labeling of STb3-27.** Synthetic peptide was radio-labeled with ¹²⁵I by using Iodobeads (Pierce Chemical Co.) as described by the manufacturer. The reaction mixture consisted of 1 μ g of synthetic peptide, 1 mCi of carrier-free ¹²⁵NaI (ICN Biomedicals, Inc., Costa Mesa, Calif.), and two iodobeads in a total volume of 0.25 ml. The reaction was started by the addition of beads and was allowed to proceed at room temperature for 20 min. Unincorporated ¹²⁵I was removed from the peptide fraction by BioGel P2 (Bio-Rad Laboratories, Richmond, Calif.) gel filtration chromatography. Radioactivity was determined by gamma-radiation spectroscopy. Void volume fractions containing radiolabeled peptide were collected and saved for incorporation into the coupling reactions described above.

(iii) **Immunization of rabbits with STb3-27-KLH.** Female New Zealand White rabbits were immunized with the STb-peptide KLH conjugate by the following regimen. Each rabbit received 1 mg (peptide equivalent based on coupling efficiency to KLH) of peptide KLH conjugate in Freund complete adjuvant. One half of the immunogen was given as approximately eight subcutaneous injections along the back; the other half was administered by intramuscular injection in a hind quarter. Four weeks following the primary immunization, rabbits were immunized with the same dose of immunogen emulsified with Freund incomplete adjuvant. A second booster immunization, which was identical to the first one, was given 1 month following the first booster immunization. Rabbits were bled, and sera were collected 1 week following the administration of the second booster immunization. Whole serum was fractionated by ammonium sulfate precipitation (33% saturation) followed by dialysis. The dialyzed immunoglobulin G (IgG)-enriched fraction was then filtered through a synthetic peptide-Sepharose 6B column prepared as described below. The eluted antibody fraction was assayed for protein by the method of Bradford (3) by using protein dye reagent obtained from Pierce Chemical Co. Aliquots were frozen (-20°C) until they were used in the ELISA method described below.

(iv) **Preparation of affinity-purified anti-STb3-27 antibody.** The STb3-27 peptide-Sepharose 6B column was prepared by reacting purified synthetic peptide with hydrated and washed epoxy-activated Sepharose 6B (Pharmacia LKB Biotechnol-

ogy, Inc.) at pH 10.5, as described by the manufacturer. The reaction proportions were 25 mg of STb3-27 to 5 g of epoxy-activated Sepharose 6B. The immobilized peptide was poured as a slurry into a column (1 by 15 cm) through which the IgG-enriched serum fractions were passed. Non-specific antibodies and other contaminating serum components were removed by extensive washing until the A_{280} profile of the column effluent returned to the base line. Peptide-specific antibody was released from the column by washing the column with NaCl (0.2 M)-glycine (0.5 M; pH 3.0). The eluted antibody preparation was neutralized, and buffer was exchanged by gel filtration chromatography over Sephadex G-25 (Pharmacia LKB Biotechnology, Inc.) equilibrated in 0.2 M phosphate buffer. Following equilibration, the protein concentration of the antibody preparation was determined (3) and aliquots were stored at -20°C until use.

STb-specific ELISA. Culture filtrates of STb-producing strains or highly purified STb (S. C. Whipp, unpublished data) were prepared for ELISA by dilution in 0.1 M carbonate buffer, pH 9.6 (dilution buffer), as follows. Samples (0.1 ml) were added to wells of 96-well Pro-Bind assay plates (Falcon no. 3915) that already contained 0.1 ml of dilution buffer; after mixing with a multichannel automatic pipettor, the samples were diluted, by serial twofold dilution, down the plate. The plates were incubated overnight at 4°C or for 2 h at 37°C to allow the STb antigen to bind. Following binding, the plate contents were dumped and the wells were then filled with blocking solution (3% gelatin in PBS) for 30 min at room temperature. The plate contents were again dumped, and the wells were rinsed once with PBS containing 0.05% Tween 20 (PBS-Tween). Primary antibody (100 μl), which was diluted appropriately in PBS-Tween, was added to the rinsed wells, which were covered with plastic film tape and incubated for 1 h at 37°C . The well contents were dumped, and the wells were then rinsed four times with PBS-Tween. Secondary antibody, goat anti-rabbit IgG alkaline phosphatase conjugate (Promega, Madison, Wis.), was diluted (1:2,000) in PBS-Tween and was added to the wells, which were then covered and incubated as described above for primary antibody. After incubation, the plate contents were dumped and the wells were rinsed as described above. Alkaline phosphatase substrate (*p*-nitrophenyl phosphate) dissolved in AP buffer (Bio-Rad Laboratories) was added to the wells, and the plates were incubated at 37°C for 1 h. Following incubation, the reaction was stopped by the addition of 0.1 ml of 1 M NaOH, and the A_{405} was then read in an automatic ELISA reader. The ELISA data are presented as the means of at least four determinations \pm 1 standard deviation. Quantitative comparisons between sets of ELISA data from single groups of experiments were accomplished by performing first-order regression analyses on the data and assigning an absorbance value (A_{405}) of 0.5 as an arbitrary ELISA titer unit. The ELISA titer was therefore the reciprocal of the dilution which yielded an A_{405} of 0.5.

Construction of hyperproducing STb plasmids. A restriction enzyme site search of the cloned STb gene contained on pCHL6 revealed that digestion of pCHL6 with *MnII* and *HindIII* would produce a 343-bp fragment that lacked the proposed -10 and -35 sequences of the STb gene but preserved the ribosomal binding and translational initiation sites and the entire STb structural gene. The *MnII-HindIII* fragment was first cloned into pT7-5 that was previously digested with *SmaI* and *HindIII*. The resulting plasmid, pUD1, is a bacteriophage T7-promoted expression plasmid for STb. A *lac*-promoted version of STb was constructed by

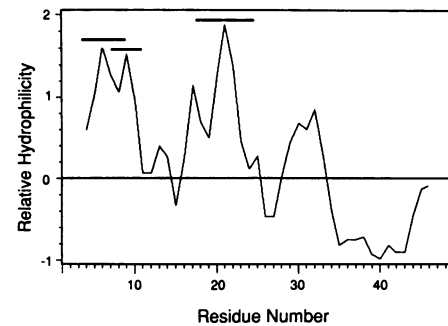


FIG. 1. Antigen profile of the secreted form of STb. The antigen plot was performed by using the PC-GENE software package (Intelligenetics, Inc., Mountain View, Calif.). Peak hydrophilic residues are indicated by bold horizontal bars and are described in the text.

digesting pUD1 with *EcoRI* and *HindIII* and, following gel purification of the STb-encoding fragment, ligation of the STb cassette into pUC18 that was previously digested with the same enzymes. The hybrid *trp-lac* (*tac*) promoter (25) was also used to promote STb synthesis. The *tac*-promoted STb encoding plasmid (pPD21; see Fig. 3) was constructed by first removing the *tac* promoter from pDR540 by digestion with *HindIII* and *BamHI* and ligating the isolated fragment into pBR322 that was previously digested with the same enzymes. This intermediate plasmid, pPD20, was then digested with *BamHI*, and the 4-bp overhang was filled in by incubation with the four deoxynucleotide triphosphates and the Klenow fragment of *E. coli* DNA polymerase I. The STb-encoding *EcoRI-HindIII* fragment of pUD1 was blunted as described above and then ligated with similarly treated vector DNA. Following transformation, clones harboring the STb gene in the correct orientation were selected by restriction analysis. These clones were then analyzed for STb expression as determined by the ELISA described above.

Chemicals and reagents. Unless specified otherwise, chemicals and other reagents were of the highest quality obtainable from common commercial sources.

RESULTS AND DISCUSSION

In a recent study (18), we examined the ability of the T7 bacteriophage expression system (30) to selectively express STb and, in pulse-chase experiments, define the mode of STb export and the position of the STb signal peptide cleavage. These data demonstrated that the 71-amino-acid pro-STb is cleaved at the inner membrane upon export to the periplasm and subsequent secretion of a 48-amino-acid extracellular STb. Thus, Ser-24 is the amino terminus of mature STb. Using these data, we analyzed the exported (mature) form of the STb protein primary structure for potential antigenic sites by the method of Hopp and Woods (15). The result of the antigen profile (Fig. 1) suggests that STb has three potential antigenic determinants based on sites of greatest relative hydrophilicity (denoted Ah). The peak hydrophilic site ($A_h = 1.88$) corresponded to residues 18 to 23. The second and third highest values ($A_h = 1.62$ and 1.53), although not reliably in or adjacent to an antigenic site (33% chance of being an incorrect site [15]), were both located in the amino-terminal portion of STb (residues 3 to 8 and 6 to 11, respectively). In an attempt to include in a synthetic STb peptide as many potential determinants as

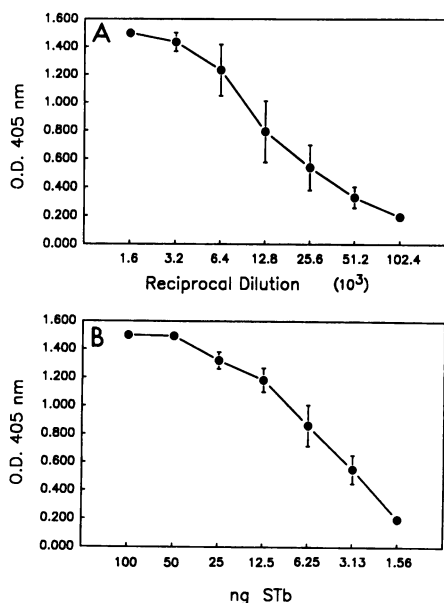


FIG. 2. STb ELISA. The titration of anti-STb3-27 antibody against bound peptide (2 μ g per well) (A) was performed in quadruplicate as described in the text. The STb standard curve (B) was performed in quadruplicate as described in the text by using a 1:1,000 dilution of anti-STb3-27 antibody.

possible, we synthesized the peptide QSNKKDLCEHYRQ IAKESCCKGFLG, which corresponded to STb3-27 (STb codons 26 to 50). The peptide was coupled to KLH and was used to generate an immune response to native STb, as described in Materials and Methods.

STb ELISA. The antibody response to KLH-coupled synthetic peptide was determined by testing dilutions of affinity-purified antibody against bound peptide in a direct ELISA. The affinity-purified antibody (5 mg/ml) still exhibited a strong reaction with bound peptide at a dilution of 1:25,000 (or 200 μ g/ml) (Fig. 2A). The ELISA reaction with bound KLH was negligible (data not shown). The specificity and sensitivity of the assay for STb was tested by performing the ELISA, at antibody saturation (1:500), on a preparation of highly purified STb (Whipp, unpublished data). The data (Fig. 2B) demonstrated that the peptide-specific antibody detected as little as 2.5 ng of STb based on first-order regression analysis of the curve and an arbitrary absorbance of 0.5. The STb3-27 antibody did not cross-react with *E. coli* STa (data not shown).

Alternate promoter hyperexpression of STb. In a previous study, Spandau and Lee (28) demonstrated that transcription of STb is controlled by a relatively weak constitutive promoter. In an attempt to overproduce STb, we replaced the endogenous STb promoter with promoters known to be highly active in *E. coli*. The expression plasmids (Fig. 3) were transferred to *E. coli* hosts and tested for their ability to express STb in defined-medium cultures. ELISA data on the production of STb by *E. coli* strains which harbored the three expression constructs and pCHL6 (the cloned copy of STb bearing its native promoter) indicated that a significant improvement in the level of STb produced by pCHL6 was attained with all three expression constructs (Fig. 4). All three of the alternate promoter systems yielded filtrates with significantly more STb than pCHL6 (Fig. 4). By performing a first-order regression on the data shown in Fig. 4 and arbitrarily assigning an absorbance of 0.5 as a reference

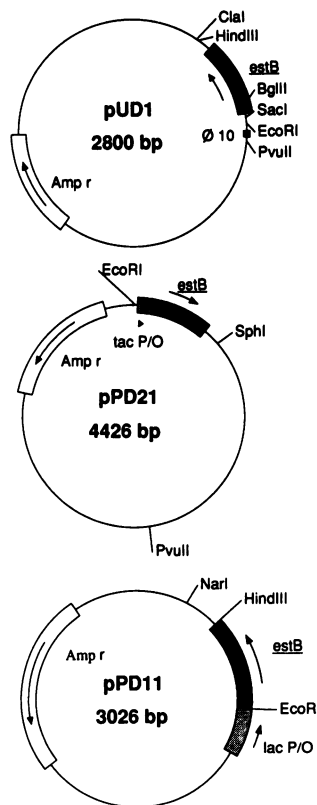


FIG. 3. STb expression plasmids. Construction of the STb expression plasmids is described in the text. Abbreviations: *estB*, STb gene; lac P/O, β -galactosidase promoter; tac P/O, *trp-lac* hybrid promoter; ϕ 10, bacteriophage T7 promoter; Amp^r, β -lactamase.

point, it was revealed that the titer for STb from the pCHL6 culture was approximately 5. Titers for cultures of the strains harboring pPD11, pUD1, and pPD21 were 52, 56, and 110, respectively. This represents increases over the amount of STb produced by the cloned *estB* gene of 10.4-, 11.2-, and 22-fold, respectively. These data were corroborated by pig intestinal loop results, which were consistent with the ELISA determinations (Table 1). In addition, it was observed that naturally occurring STb-producing isolates make very little STb (10 to 20% of that produced by pCHL6) in defined medium (data not shown).

Spandau and Lee (28) determined that the native STb promoter is weak relative to those of the *lacZ* or *ompF* operons. They suggested that the 2-log-unit reduction in STb

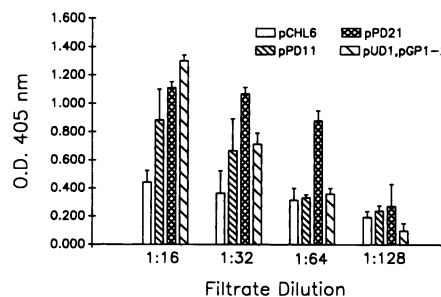


FIG. 4. Comparative production of STb by strains harboring recombinant expression plasmids. Culture filtrates of the various strains were prepared, diluted as shown, and assayed by the ELISA described in the text.

TABLE 1. STb production by selected recombinant strains

Strain	Dilution ^a	Loop vol/length (ml/cm) ^b
HB101(pCHL6)	Undiluted	0.267 ± 0.06
	1:5	0.103 ± 0.03
	1:25	0.078 ± 0.01
	1:125	0.008 ± 0.01
HB101(pPD21)	Undiluted	0.425 ± 0.05
	1:5	0.307 ± 0.04
	1:25	0.178 ± 0.01
	1:125	0.087 ± 0.01

^a Culture filtrate dilution.

^b There were six loops per dilution.

transcripts in vitro, relative to *lacZ*, might be explained by the occurrence of a G residue at the last position of the STb Pribnow heptamer (20). Replacement of the endogenous STb promoter with one with a higher relative activity, like that of *lacZ*, increases the number of STb transcripts within the cell and should therefore increase the level of STb made by that cell. There are several additional factors, however, that do not permit a strict conversion of transcript number to protein product. Factors that can influence the efficiency of translation include message stability, ribosomal binding to the message, and 5' secondary structures that may affect the rate of translation initiation. In addition, proteins destined for export are processed at a finite rate independent of transcription and translation. It is therefore not surprising that, although *lacZ*, *tac*, and T7 promoters are potentially 2 to 3 log units more active than the STb promoter (28), we observed increases in the amount of extracellular STb that were only 10- to 20-fold greater than that of a strain harboring a cloned copy of STb. It should be noted that gene copy number would not play a role in our determinations since all of the vectors possessed the same origin of replication. The significant increase in STb produced by the strain harboring pPD21 may reflect more efficient translation of the *tac*-promoted STb message since the promoter construct used possessed a tandem set of Shine-Dalgarno sequences positioned just 5' to the native STb ribosomal binding site. This arrangement may provide for more efficient ribosomal loading of that particular STb message. Since our goal in this effort was to construct plasmids that would increase the yield of STb from a defined-medium culture, we addressed the amount of STb that gets outside of the cell. Development of the ELISA described here will allow us to focus on growth conditions and medium components that may optimize extracellular toxin yields.

In addition to our goal of hyperexpression of STb, another principal goal of this investigation was to develop an easily reproducible ELISA detection method for the toxin. By using such an assay, an evaluation of STb production and recovery could readily be determined, thus eliminating the necessity for routine use of costly and laborious intestinal loop assays. Previous investigators (13) described a competitive ELISA for STb using antibodies raised against a protein A-STb recombinant fusion protein to detect native STb competed with a recombinant STb-β-galactosidase protein fusion antigen. The assay was sensitive but impossible to reproduce without access to (or reconstruction of) the recombinant strains, and furthermore, the results, as described, were difficult to interpret from a quantitative standpoint. In designing our assay, we hoped to provide the basic information necessary to reproduce the assay in almost any

research laboratory. The major requirement for the assay reported here is the initial synthesis of a synthetic peptide that spans the major antigenic region of STb. Although we have no experimental evidence as yet, it is entirely likely that immunization with a significantly smaller peptide that spans the major predicted antigenic site (Lys-18 to Lys-23) would yield comparable results. This would greatly reduce the initial expense of reproducing the assay as described here. Although not shown here, the assay can be reproduced by using an IgG fraction of immune serum or even whole serum instead of affinity-purified antibody. The risk of spurious cross-reaction with other antigens in the assay mixture could present problems; however, the use of synthetic peptides for immunogens reduces the potential for the generation of cross-reactive antibodies, provided that the pre-immune sera from selected animals are substantially free of cross-reactive antibodies to *E. coli* products. Affinity purification of the peptide-specific antibody does, however, provide a degree of specificity that otherwise cannot be attained without the use of monoclonal antibodies.

Aside from the obvious advantage of the ELISA described in this report over intestinal loop assays, there is a considerable degree of reproducibility that cannot be achieved with bioassays in general. Factors such as variability in animals, location of sample loops within the gut, and neighboring loop contents all contribute to variations in assay results that may translate to as much as three- to fivefold differences in toxin concentration. This variability complicates quantitative toxin assessments, and although the tendency is to regard these differences as being within the limits of precision, this level of variation is unacceptable when evaluating the efficiency of a purification regimen. Use of the ELISA described here will dramatically shorten the time and cost of STb purification protocols, and at the same time, it will permit assay of many more individual fractions. This undoubtedly will result in significant improvements in our schemes to produce large quantities of highly purified STb.

ACKNOWLEDGMENTS

R.G.U. is a James W. McLaughlin Predoctoral Research Fellow. This research was supported by the James W. McLaughlin Fund, The University of Texas Medical Branch, Galveston.

We thank John W. Peterson for critical review of the manuscript.

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