Production of *Escherichia coli* STb enterotoxin is subject to catabolite repression

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Enterotoxigenic *Escherichia coli* are known to secrete several types of toxins including STb, a heat-stable enterotoxin. STb enterotoxin production was studied in wild-type *E. coli* strains. Using a quantitative STb-specific inhibition ELISA, the amount of toxin present in the culture supernatant fractions of various *E. coli* strains was determined. Variation in the production of STb toxin was observed for the wild-type strains. For *E. coli* strain 82-4247 grown in trypticase soy broth, the toxin was produced after 4 h of growth and was maximal after about 57 h of growth. The amount of toxin in the culture supernatant fraction increased concomitantly with bacterial growth. Using the rat loop assay, the biological activity of STb was retained even after the logarithmic phase of growth when STb production levelled off (i.e. from 24 to 74 h). STb production by *E. coli* strain 82-4247 varied with the culture medium used. In particular, addition of 10% (w/v) compared to 0.1% glucose to Davis minimal medium decreased STb production, whereas addition of 1.0% (w/v) glycerol did not affect STb production. Addition of exogenous cAMP reversed the repressive effect of glucose. Using mutant strains, STb production was shown to be subject to catabolite repression.

**Keywords**: enterotoxigenic, *Escherichia coli*, STb enterotoxin, catabolite repression

**INTRODUCTION**

Enterotoxigenic *Escherichia coli* (ETEC) strains are known to cause severe diarrhoea in humans and animals by production of heat-labile and heat-stable enterotoxins (Mainil et al., 1986; Söderlind et al., 1988). Two types of heat-stable enterotoxins are secreted by porcine *E. coli*. The first type, STa or STI, is methanol-soluble and induces intestinal secretion in infant mice and neonatal pigs (Burgess et al., 1978). The second type, STb or STII, is methanol-insoluble and induces intestinal secretion in weaned and neonatal pigs but does not induce secretion in infant mice (Weikel et al., 1986). STb is considered an important cause of diarrhoea in pigs (Mainil et al., 1986; Moon et al., 1986), but is rarely associated with humans (Echeverria et al., 1984; Guerrant et al., 1990; Lortie et al., 1991a; Handl & Flock, 1992). STb was found to be sensitive to protease degradation (Whipp, 1987) as addition of protease inhibitor to STb samples led to the development of intestinal loop assays using animals other than pigs (Dubreuil et al., 1991; Whipp, 1991; Hitotsubashi et al., 1992).

The *estB* gene, encoding STb, was cloned and sequenced (Lee et al., 1983; Picken et al., 1983); it encodes a 71 amino acid polypeptide, including a 23 amino acid signal sequence (Kupersztoch et al., 1990; Fujii et al., 1991). In the periplasm the toxin is found as an 81 kDa precursor that is then converted to, and secreted as, a 52 kDa mature protein (Kupersztoch et al., 1990). STb enterotoxin is not associated with the cellular fraction but found preferentially in the culture supernatant fraction (Handl & Flock, 1990; Kupersztoch et al., 1990).

One obstacle in STb research is the poor yield of toxin from wild-type strains. Spandau & Lee (1987) demonstrated that transcription of *estB* gene is controlled by a relatively weak promoter that accounts for the low toxin production. Urban et al. (1990) developed a sensitive ELISA to quantify STb production and showed that wild-type STb-producing strains made little STb in defined medium. In this paper, using a quantitative ELISA, the kinetics of STb production and STb production in different culture media were studied. Catabolite repression of STb production was specifically examined.

**METHODS**

**Bacterial strains and media.** Porcine ETEC wild-type strains used in this study were from our collection (Dubreuil et al.,...
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1991). E. coli strain HB101 [F- hsdS20 (r, m) recA13 ara-14 proA2 lacY1 galK2 rpsL20 (Sm') xyl-5 mit-1 supE44 Δ (Xyl ') (Boyer & Rouillard-Dussoix, 1969). E. coli cAMP receptor-protein-defective strain TP2139 (F- syl crp-39 lacA74 argH1) (Wang et al., 1993), and E. coli adenylate-cyclase-defective strain TP610 (F- cya-610 thi-1 thr-1 knb6 lacY1 tonA21 supE44 hsdR hsdM recBC lup-11 lig4 Δ (Hedegaard & Danchin, 1985) were also used. Selective pressure was imposed on strains harbouring plasmid pRAS1 by addition of 50 µg ampicillin ml-1 to the medium. All wild-type strains were tested for STb by an enhanced chemiluminescence immunodot assay (Lortie et al., 1991c), hybridization assay with a radioactive STb DNA probe, and biological assay in rat ligated intestinal loops (Lortie et al., 1991b). Strain 82-4247 (serotype O115, ‘KV165’, STa*, STb*, LT-) was used as positive control and strain 84-3922 (serotype O115, ‘KV165’, STa*, STb*, LT-) was used as the negative control. The following liquid media were used: Luria-Bertani broth (LB, 10 g tryptone, 4.8 g yeast extract, 10 g NaCl l-1); Luria-Bertani broth (LB, 10 g tryptone, 4.8 g yeast extract, 10 g NaCl l-1); Davis minimal medium (DMM, Difco), brain heart infusion broth (BHI, Difco); trypticoye extract broth (TSB, BBL) and trypticoye yeast extract broth (TYEB) (10 g tryptone, 5 g yeast extract, 5 g NaCl, 2 g glucose l-1 water). Strains in each liquid medium were incubated at 37 °C with agitation (150 r.p.m.), and after 18 h of growth, 50 µl of the culture was transferred to broth (LB, 10 g tryptone, 4.8 g yeast extract, 10 g NaCl l-1). Plasmid and DNA manipulations. The recombinant plasmid pRAS1 (Whipp et al., 1986), containing the genetic determinant encoding STb enterotoxin was used in recombinant strains. This plasmid consisted of an insert of a 1.2 kb HindIII restriction fragment from the enterotoxin plasmid P307 (Gyles et al., 1974) into plasmid pBR322. The pRAS1 plasmid DNA was purified by alkaline lysis (Maniatis et al., 1982). Electromembranations of pRAS1 into strains HB101, TP610 and TP2139 were done with a gene pulser (Bio-Rad) (Bosse et al., 1993).

Production and purification of E. coli heat-stable enterotoxin. To obtain pure STb toxin to set up the quantitative ELISA, an E. coli strain HB101 bearing the gene fusion (estB malE) plasmid construct called pMAL-STb, described earlier by Bossé et al. (1993), was used. This construct is under the control of P, and can be induced by IPTG. Briefly, from the recombinant strain, an osmotic shock fluid was obtained and the fusion protein MBP-STb was purified using affinity chromatography (amylose column) (Handl et al., 1993). The MBP-STb fusion protein was then cleaved with protease Xa (New England Biolabs). The cleaved material was loaded on a Poros R2/H reverse-phase column (Perspective Biosystems) and eluted with acetonitrile. The purified STb toxin was lyophilized and kept at -20 °C.

STb antiserum. The methods for production of Staphylococcus aureus protein A-STb fusion protein and immunization of rabbits and analysis of sera done according to Handl et al. (1988). Briefly, rabbits were immunized with 50 µg fusion protein in Freund’s adjuvant (Sigma) in a 1:1 ratio. Injections were given subcutaneously at six to eight places. A booster dose of 50 µg was given after 4 weeks. Serum was collected 4 weeks after the last injection and kept frozen at -20 °C until used.

STb-specific inhibition ELISA. Flat-bottom polystyrene microtitre plates (Nunc Immunoplates 4-39454, Copenhagen, Denmark) were coated at 4 °C for 18 h with 25 ng pure STb in 100 µl coating buffer (10 mM phosphate buffer, pH 7.4) per well. Then, 100 µl coating buffer containing 0.5% (w/v) casein and 0.05% (v/v) Tween 20 was added and left 1 h at room temperature to block free sites. The plates were washed three times with 0.05% (w/v) saline containing 0.05% (v/v) Tween 20 (saline-Tween). A 100 µl volume of the sample was added to each well and incubated at room temperature for 1 h. The sample consisted of 50 µl antibodies raised against Protein A-STb fusion protein (Handl et al., 1988) diluted 1:500 in coating buffer with 0.05% (v/v) Tween 20 (PBS-Tween) and 50 µl cell-free culture supernatant fraction of the tested strains. This solution was pre-incubated for 15 min at room temperature. The cell-free culture supernatant fraction was obtained by filtration through low-protein-binding 0.22 µm membrane filters (Gelman Sciences). The contents of the wells were discarded and the plates were washed three times with saline-Tween. Goat anti-rabbit IgG conjugated to alkaline phosphatase (Jackson Immunoresearch) was diluted 1:4000 in PBS-Tween and 100 µl of the diluted conjugate was added to each well and incubated at room temperature for 1 h. The contents of the wells were discarded, the plates were washed three times with saline-Tween and 100 µl of the substrate p-nitrophenyl phosphate (Sigma), dissolved in substrate buffer (1 M diethanolamine buffer and 0.05 mM MgCl2, pH 9.8), was added to each well (Hawkes, 1982). The plates were incubated at 37 °C and read at 410 nm using an automatic plate reader (model MR5000, Dynatech Laboratories) when the minimum inhibition control consisting of 100 µl PBS-Tween reached 1.0. The maximum inhibition control consisted of 100 µl protein A-STb antibodies diluted 1:1000 in PBS-Tween. All samples were tested in triplicate.

To test the specificity of the ELISA, a cell-free culture supernatant fraction of strain B94-2361A1 [STa* (porcine STa), STb* and thermostable toxin (LT*)] and 10 µg purified porcine LT ml-1 (Sigma) in TSB were tested. Data were expressed in Fig. 2 as µg ml-1 so comparison with other enterotoxins could be made. However, since bacterial growth varied with the culture medium used, STb enterotoxin production was then expressed as µg (mg cell dry weight)-1. Cell dry weight was measured by drying in an oven (105 °C), to constant weight, the cells from a 10 ml volume of a bacterial culture. Those cells were obtained by centrifugation at 15000 g for 15 min and washed twice in saline before drying.

Kinetics of STb production. E. coli strains were inoculated in TSB and incubated for various periods of time. Determination of c.f.u. was by dilution and plate counting on blood agar plates [tryptic soy agar II (BBL) + 5% (v/v) bovine blood].

Rat loop assay. The rat loop assay was done as described by Dubreuil et al. (1991). Briefly, 6- to 8-week-old white rats (Sprague Dawley) were used. The rats were fasted for 48 h and their abdomens were incised. A volume of 20 mM Tris/HCl buffer (pH 6.8) containing 300 µg soybean trypsin inhibitor ml-1 (80 µl) (Boehringer Mannheim) was injected into the small intestine and left for 5 min. The liquid was removed and eight loops of 5 cm each were made. Aliquots (500 µl) were injected into each loop. After 4 h, the rat was euthanized and the volume of liquid, the length and the circumference of each loop was measured. Results were expressed as the volume of liquid (ml) per length (cm) x circumference (cm) of the intestine. A ratio of greater than 0.05 was considered positive. Each sample was tested twice in different animals.

Effect of mitomycin and lincomycin. TSB containing 2, 1 or 0.5 µg mitomycin C ml-1 (Sigma) or 150, 75, 37.5 or 18.8 µg lincomycin ml-1 was inoculated with E. coli strains 82-4257 or 48-3922 and incubated at 37 °C for 18 h.

Effect of glucose and cAMP. E. coli wild-type strains and strains HB101, TP610 and TP2139, with or without pRAS1, were inoculated in DMM containing 0.1% or 1% glucose (w/v) with or without 1 mM cAMP (Sigma). Strain 82-4257 was also grown in the same medium containing 1% glycerol (w/v).
STb is catabolically repressed with or without 1 mM cAMP. Strains HB101 and HB101 (pRAS1) were inoculated in DMM plus Casamino acids (250 μg ml⁻¹).

RESULTS

This aim of this study was to improve the understanding of STb enterotoxin production by E. coli. To this end a quantitative STb-specific inhibition ELISA was first developed using purified STb toxin and polyclonal antibodies raised against a Protein A-STb fusion protein. A standard curve using varying amounts of purified toxin was constructed. Based on first-order regression analysis of the standard curve, the amount of STb in each sample was evaluated (Fig. 1). Values less than or equal to 0.02 ± 0.01 μg ml⁻¹ were obtained with all four STb- strains, including porcine STa (one strain) and porcine LT (a purified LT preparation) controls, and thus were considered negative values for STb production.

A kinetic study of STb production in TSB using strain 82-4247 showed that production was observed after 4 h of incubation (Fig. 2). The amount of toxin in the culture supernatant fraction increased concomitantly with growth and was maximal after about 57 h of growth. At that point, the toxin level reached a plateau and after 74 h of incubation it remained about the same. From 24 to 74 h of growth, the culture supernatant fraction of strain 82-4247 was tested for biological activity using the rat loop assay. At 33 and 74 h of growth the toxin was active with intestinal ratios of 0.200 ± 0.02 ml cm⁻² and 0.210 ± 0.04 ml cm⁻², respectively.

![Fig. 1. STb-specific inhibition ELISA standard curve. Results are the means ± SD of ten different experiments.](image)

![Fig. 2. Growth and STb production by two porcine strains of E. coli. Strains were grown in 500 ml TSB at 37 °C and shaken at 150 r.p.m. Results are the means ± SD of two different experiments. OD₆₅₀ of strain 82-4247 (□) and strain 84-3922 (△). STb production by strain 82-4247 (■) and strain 84-3922 (▲).](image)

The effects of lincomycin and mitomycin C in sub-inhibitory concentrations as inducers of toxin production was studied. For strain 82-4247 in the presence or the absence of both inducers, no difference in STb production was noted compared to controls [on average 3.8 ± 0.1 μg (mg dry wt)⁻¹].

STb production by various porcine E. coli wild-type strains was quantified. The production in TSB was determined in cell-free culture supernatant fractions after 18 h of growth. Because growth differed with the strain and the culture medium used, for comparison purposes, all data were expressed as μg toxin (mg dry wt)⁻¹. STb⁺ strains produced 0.8–3.8 μg (mg dry wt)⁻¹; strain 82-4247 produced the highest level of toxin (Table 1).

<table>
<thead>
<tr>
<th>Strain*</th>
<th>STb production [μg (mg dry wt)⁻¹]†</th>
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<tbody>
<tr>
<td>81-550</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>85-880</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>82-4247</td>
<td>3.8 ± 0.1</td>
</tr>
<tr>
<td>79-5863</td>
<td>2.0 ± 0.01</td>
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</table>

* Grown at 37 °C for 18 h in 5 ml TSB shaken at 150 r.p.m.
† Data determined with STb-specific inhibition ELISA. Results are the means ± SD of three different experiments.
Next, production of STb in different culture media was evaluated. Complex and minimal medium were compared to determine STb synthesis. The amount of STb toxin produced varied with the culture medium used (Table 2). In LB broth, strain 82-4247 produced 6.6 μg (mg dry wt)\(^{-1}\) compared to 0.3 μg (mg dry wt)\(^{-1}\) in TYE broth.

Table 2. Quantitative evaluation of STb production by a porcine E. coli strain 82-4247 in various culture media

<table>
<thead>
<tr>
<th>Medium*</th>
<th>STb production [μg (mg dry wt)(^{-1})]†</th>
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<tbody>
<tr>
<td>Trypticase soy broth</td>
<td>3.8 ± 0.1</td>
</tr>
<tr>
<td>Brain heart infusion</td>
<td>3.1 ± 0.02</td>
</tr>
<tr>
<td>Tryptone yeast extract broth</td>
<td>0.3 ± 0.01</td>
</tr>
<tr>
<td>Davis minimal medium</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Luria–Bertani broth</td>
<td>6.6 ± 0.1</td>
</tr>
</tbody>
</table>

* Growth was at 37 °C for 18 h in 5 ml culture medium shaken at 150 r.p.m.
† Data determined with STb-specific inhibition ELISA. Results are the means ± SD of three different experiments.

Since the difference in the compositions between these two media is the glucose concentration, we investigated the effects of glucose on the production of STb enterotoxin using a minimal medium. Wild-type strain 82-4247 and strain HB101 (pRAS1) produced more STb enterotoxin when 0.1 % compared with 1.0 % glucose was added to DMM (Table 3). Upon addition of 1 mM cAMP to the culture medium the amount of toxin produced was increased in the presence of either 0.1 or 1.0 % glucose. This phenomenon was not observed when cells were grown in 1.0 % glycerol. The repressive effect of glucose was also observed with the wild-type strain 81-550 (data not shown).

STb production by the cya and crp mutant strains was lower than that observed with strain HB101 (pRAS1). The presence of exogenous cAMP increased STb production markedly for HB101 (pRAS1) and the cya strain while the crp mutant strain was not affected. A DNA homology search revealed that a sequence showing 72 % identity with the 22 bp consensus CRP-binding site was present in the nucleotide sequence 26 bp upstream of the −35 region of the transcriptional start site of the estB gene (Fig. 3).

DISCUSSION

Study of enterotoxin STb production has been difficult owing to the fact that tools for toxin detection have only recently become available. Another difficulty with the study of STb toxin has been the lack of information on conditions required for its production. Despite only sparse information on growth conditions and culture medium components that could increase STb production being available, STb toxin was purified from wild-type E. coli (Dubreuil et al., 1991) and from genetic constructs (Fujii et al., 1991; Dreyfus et al., 1992; Handl et al., 1993).

Table 3. Quantitative evaluation of STb production by E. coli strains, in DMM supplemented with glucose and cAMP

<table>
<thead>
<tr>
<th>DMM supplemented with:</th>
<th>STb production [μg (mg dry wt)(^{-1})]* by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>82-4247</td>
</tr>
<tr>
<td>0.1 % Glucose</td>
<td>3.5 ± 0.03</td>
</tr>
<tr>
<td>1.0 % Glucose</td>
<td>2.1 ± 0.04</td>
</tr>
<tr>
<td>0.1 % Glucose + 1 mM cAMP</td>
<td>20.1 ± 2.8</td>
</tr>
<tr>
<td>1.0 % Glucose + 1 mM cAMP</td>
<td>12.6 ± 0.1</td>
</tr>
<tr>
<td>1.0 % Glycerol</td>
<td>8.8 ± 0.03</td>
</tr>
<tr>
<td>1.0 % Glycerol + 1 mM cAMP</td>
<td>7.1 ± 1.2</td>
</tr>
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</table>

* Data determined with STb-specific inhibition ELISA. Results are the means ± SD of three different experiments.
Our laboratory overexpressed STb using gene fusion technology and purified the toxin to homogeneity in large quantities (Handl et al., 1993). Pure STb toxin was used to set up an STb-specific inhibition ELISA to quantitatively evaluate STb production. The use of an inhibition assay over a direct or an indirect ELISA allowed the evaluation of STb production in different culture media without significant interference from medium components. It has been shown for STa (Alderette & Robertson, 1977b) and for STb (Kupersztoch et al., 1990; Handl & Flock, 1990) that these heat-stable enterotoxins are secreted extracellularly. No significant STa or STb activity was found to be cell-bound and these toxins were not stored within the periplasmic space. Thus, in our study, cell-free culture filtrates solely were assayed for STb production. As Moon et al. (1986) have shown that many ETSC isolates carry more than one enterotoxin gene, it was important to determine the specificity of our ELISA. No cross-reaction with porcine STa or porcine LT toxins was noted.

Strain 82-4247 produced the highest levels of STb of all tested wild-type strains. Using the intestinal rat loop assay, we had previously observed that fluid accumulation (ml cm⁻²) differed depending on the E. coli strains tested (Dubreuil et al., 1991). In this same study, using this biological strain, strain 82-4247 was among the best STb producers of the wild-type strains tested. Spandau & Lee (1987) have proposed that STb is expressed via a weak promoter; the observed differences in STb production might be accounted for by different levels of gene expression.

The kinetics of STb production in a complex medium were examined. The results showed that the amount of toxin in the culture supernatant fraction increased concomitantly with growth. For strain 82-4247, STb toxin production reached a plateau after 57 h of growth. Significant biological activity was observed during this growth phase. This suggested that either the toxin produced was stable under the conditions found in the culture supernatant fraction and was not degraded by proteases, or that active STb was synthesized in equivalent amount to that which was being degraded.

Production of STb enterotoxin was not altered by addition of sub-inhibitory concentrations of either lincomycin or mitomycin C. Levner et al. (1977) and Alderette & Robertson (1977b) showed that a protein synthesis inhibitor such as lincomycin, when added in sub-inhibitory concentrations to the culture medium, increased the rate and duration of both Vibrio cholerae and E. coli LT toxin synthesis. It was suggested that toxin production was limited by one or more protein factors, whose synthesis is particularly sensitive to lincomycin (Levner et al., 1977). Recently, Al-Jumaili et al. (1992) reported an increase in Vero cytotoxin production when mitomycin C was added to the culture medium. Mitomycin C is known to be a non-specific inducer for phage- and plasmid-encoded proteins (Reeves, 1972).

The higher level of production of STb in a medium (LB) containing no glucose compared to one containing 1% glucose (TYEB) suggested that production of STb was repressed by glucose. Alderette & Robertson (1977a) reported a similar observation for STa and Amirmozafari & Robertson (1993) for Yersinia enterocolitica toxins. Glycerol as the sole source of carbon did not repress STb production. Addition of cAMP to the medium increased STb production, indicating that expression of lower amounts of STb toxin in presence of glucose is due to catabolite repression.

Using mutant strains which are deficient for crp and cya, catabolite repression of STb enterotoxin was confirmed. It is known that the cAMP-CRP regulation mechanism mediates expression of certain E. coli virulence factors, for example, colonization factor antigen II (Evans et al., 1991), STa enterotoxin (Alderette & Robertson, 1977a) and the Pap pilus (Göransson et al., 1989). Catabolite repression is due to the inactivation of adenylate cyclase when glucose is transported into the cell (Adhya & Garges, 1982). Adenylate cyclase produces cAMP from ATP and is not active in the presence of glucose. In enteric bacteria, genes whose transcription is subject to catabolite repression are positively regulated by cAMP. The catabolite gene activator protein when complexed with cAMP binds to a specific DNA sequence named the CRP-binding site. This site occurs at different distances upstream from the transcriptional start site in different operons and leads to the activation of transcription. A DNA region showing 72% identity with the CRP consensus sequence (Gargas, 1994) was found 26 nucleotides upstream of the -35 region of the estB gene. It has been shown that the CRP-binding site is a palindromic consensus sequence that covers 22 bp; a 6 bp spacer separates two core motifs, which consist of the 5 bp sequence TGTGA (Kolb et al., 1993). The result of sequence alignment demonstrated that in estB the first core motif is less conserved than the second. In summary, our results show that STb toxin production is subject to catabolite repression.

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