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

Philippe Busque, Ann Letellier, José Harel and J. Daniel Dubreuil

Author for correspondence: J. Daniel Dubreuil. Tel: +1 514 773 8521 ext. 8433. Fax: +1 514 778 8108.

**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.,...
Enzyme-linked immunosorbent assay (ELISA) was done as described by Handl et al. (1988). 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 (8.0 ml) (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) × 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 82-4247 or 82-3922 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 0.05% (w/v) glucose (w/v) with or without 1 mM cAMP (Sigma). Strain 82-4247 was also grown in the same medium containing 1% (w/v) glyceral (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.

A quantitative evaluation of STb production in TSB by porcine STb⁺, STa⁻ and LT⁻ E. coli strains

Table 1.

<table>
<thead>
<tr>
<th>Strain*</th>
<th>STb production [μg (mg dry wt⁻¹)]</th>
</tr>
</thead>
<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>
</tr>
</tbody>
</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.

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).
Next, production of STb in different culture media was evaluated. Complex and minimal medium were compared using STb⁺ strain 82-4247. In all experiments, the STb⁻ strain 84-3922 was tested in parallel. All media used in this work supported 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.

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 by strain 82-4247. In all experiments, the STb⁻ strain 84-3922 was tested in parallel. All media used in this work supported 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>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptase 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>Trypsone 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.

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

Strains were grown at 37 °C for 18 h in 5 ml culture medium shaken at 150 r.p.m.

<table>
<thead>
<tr>
<th>DMM supplemented with:</th>
<th>STb production [µg (mg dry wt)⁻¹]* by:</th>
<th>82-4247</th>
<th>HB101 (pRAS1)</th>
<th>TP610 cya- (pRAS1)</th>
<th>TP2139 crp- (pRAS1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% Glucose</td>
<td>3.5 ± 0.03</td>
<td>8.5 ± 1.3</td>
<td>1.4 ± 0.3</td>
<td>1.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>1.0% Glucose</td>
<td>2.1 ± 0.04</td>
<td>2.8 ± 0.2</td>
<td>4.4 ± 0.9</td>
<td>0.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>0.1% Glucose + 1 mM cAMP</td>
<td>20.1 ± 2.8</td>
<td>12.2 ± 0.6</td>
<td>64.6 ± 2.5</td>
<td>1.1 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>1.0% Glucose + 1 mM cAMP</td>
<td>12.6 ± 0.1</td>
<td>3.6 ± 0.2</td>
<td>41.3 ± 1.4</td>
<td>0.9 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>1.0% Glycerol</td>
<td>8.8 ± 0.03</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>1.0% Glycerol + 1 mM cAMP</td>
<td>7.1 ± 1.2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

* Data determined with STb-specific inhibition ELISA. Results are the means ± SD of three different experiments.

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).
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 STb (Kupersztch 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 ETEC 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 assay, 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 (Garges, 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.

ACKNOWLEDGEMENTS

This study was supported in part by a grant to J.D.D. from the Natural Sciences and Engineering Research Council of Canada (OGP0139070). We thank F. Daigle for technical assistance with DNA manipulations and Dr M. Frenette, GREB, U. Laval, Canada, for providing mutant strains.

REFERENCES


Amirmozafari, N. & Robertson, D. C. (1993). Nutritional require-


Received 30 December 1994; revised 8 March 1995; accepted 27 March 1995.