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REVIEW ARTICLE

Escherichia coli STb enterotoxin

J. Daniel Dubreuil

Tel: +1 514 773 8521 ext. 8433. Fax: +1 514 778 8108. e-mail: dubreuid@ere.umontreal.ca

Département de pathologie et microbiologie vétérinaires, Faculté de médecine vétérinaire, Université de Montréal, Saint-Hyacinthe (Québec), Canada J2S 7C6

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Overview

Enterotoxigenic *Escherichia coli* (ETEC) can cause severe diarrhoea in humans and animals by production of two distinct types of enterotoxin, a heat-labile enterotoxin (LT) with two subtypes (LTI and LTII) and a family of heat-stable enterotoxins (ST) (Söderlind *et al*., 1988). These enterotoxins reversibly alter normal intestinal homeostasis, causing intestinal secretion and diarrhoea (Betley *et al*., 1986, Weikel *et al*., 1986a). LT is a high-molecular-mass toxin (85 kDa) functionally and structurally related to *Vibrio cholerae* toxin (Sprangler, 1992). STs are low-molecular-mass toxins that retain toxic activity after incubation at 100 °C for 30 min, whereas LT loses activity under these conditions (Weikel *et al*., 1986a).

In 1978, Burgess *et al*. demonstrated that two types of ST were produced by *E. coli*; one toxin was methanol-soluble and active in the intestine of infant mice (STa), the other was methanol-insoluble, inactive in infant mice, but active in ligated intestinal segments of newborn and weaned piglets (STb). At that time, heat-stable enterotoxin was used as a generic term (ST) including STa and STb. The terms STI and STII are also commonly used as synonyms for STa and STb, respectively (Handl *et al*., 1988). Although the identity of STb toxin was established almost two decades ago, only recently did it attract sufficient interest to allow recognition of a new mechanism of action distinct from that of other known toxins. In the last few years, studies on the chemical and physical features, genetics and immunogenicity of STb have shed light on this intriguing yet neglected enterotoxin.

This review aims at summarizing the current knowledge of STb. It will be limited to STb enterotoxin; for information on LT and STa enterotoxins, readers are referred to the following reviews: Li (1992); Sprangler (1992); Gyles (1994); Sears & Kaper (1996).

Clinical importance of STb

Since the discovery of STb toxin in the late 1970s, numerous studies have set out to determine its importance in diarrhoeal illnesses. Firstly, epidemiological studies were conducted to establish the role of STb in disease by relating the presence or absence of STb with diarrhoea in animals. Then, since ETEC strains must possess colonization factors that permit adhesion and colonization of the small intestinal epithelium, many studies focused on establishing a relationship between the presence of specific fimbrial adhesins and the presence of enterotoxins. Several researchers have also investigated the putative association of serotype and toxin production. In these investigations, STb was determined using DNA probes to detect, by hybridization, the presence or absence of the enterotoxin genes. The adhesins were detected and identified using either DNA probes or antibodies. STb has historically been associated mainly with ETEC of porcine origin. However, a more thorough examination of bovine, chicken and even human ETEC strains has revealed the presence of the STb gene in some of these strains also.

Porcine strains

Production of STb is principally, but not solely, associated with porcine ETEC and although STb is usually found in combination with one or several enterotoxins, a significant number of ETEC strains produce STb only. These strains were important in establishing the role of the toxin in diarrhoea. As early as 1980, Moon *et al*. demonstrated the existence of ETEC strains that were LT^-, STa^- and STb^+ (Table 1). In the same study, neonatal pigs experimentally inoculated with an LT^-, STa^- and STb^+ strain producing the colonization factor F_5 (K99) adhered to villi, intensively colonized the ileum and caused profuse diarrhoea, indicating clearly an active role of STb in animals. On the other hand, three STb-producing ETEC strains that did not seem to possess any of the tested fimbriae (F_4, F_5 and F_6, previously known as K88, K99 and 987P, respectively) were observed. One of these strains was shown to produce F_6 fimbriae after *in vivo* growth, indicating that for certain strains, fimbriae are produced only if the isolate is passaged *in vivo*.

Numerous epidemiological studies using hybridization techniques and enterotoxin gene probes were done on

Table 1. Studies on porcine STb$^+$ ETEC strains as determined with DNA probes and by the presence of diarrhoea in pigs

Reference	O-types	Fimbrial antigen				Diarrhoea*
		F_4	F_5	F_6	Type 1	
Moon *et al.* (1980)	ND	−	+	−	ND	+
de Castro *et al.* (1984)	O18, O115, O119	ND	ND	ND	ND	+
Wilson & Francis (1986)	O8, O20, O101, O141, O149, O157	+	−	+	+	+
Fairbrother *et al.* (1988)	O20	−	+	−	ND	+
Monckton & Hasse (1988)	ND	+	−	ND	ND	+
Söderlind *et al.* (1988)	ND	−	−	−	ND	−
Fairbrother *et al.* (1989)	O115	−	−	−	ND	+
Nagy *et al.* (1990)	O141, O147, O149, O157	+	−	−	ND	+
Harel *et al.* (1991)	O8, O64, O115, O138, O139, O147, O149, O157	+	+	−	ND	+
Osek & Truszczynski (1992)	ND	+	−	−	−	+
J. M. Fairbrother, personal communication	ND	−	−	−	ND	+

ND, Not determined.

* As observed with ETEC STb$^+$, STa$^-$ and LT$^-$ isolates.

large numbers of *E. coli* isolates to determine the causal association of STb with diarrhoeal illnesses. For example, Moon *et al.* (1986) showed that 74% of ETEC isolated from swine with enteric colibacillosis hybridized with the STb probe. Interestingly, 33% of ETEC from swine more than 1 week of age with enteric colibacillosis were positive for STb only. The association of STb$^+$ strains with colibacillosis was confirmed in various studies (de Castro *et al.*, 1984; Wilson & Francis, 1986; Fairbrother *et al.*, 1988; Monckton & Hasse, 1988).

Different research groups established that isolates positive for STb were also positive for other enterotoxins. The most prevalent enterotoxin gene combination observed was LT-STb (Moon *et al.*, 1986; Wilson & Francis, 1986; Nagy *et al.*, 1990; Harel *et al.*, 1991; Osek & Truszczynski, 1992). Studies of the association of STb with adhesion factors and with strain serotype were also conducted (Table 1). F_4 fimbriae were most often associated with STb$^+$ strains. STb production was observed for many *E. coli* serotypes and its production was not restricted to particular serotypes.

Other studies revealed the existence of *E. coli* STb$^+$ strains for which no adhesion factors were found. Two of these strains were used to inoculate newborn piglets that had suckled from their mother and no symptoms were induced (Söderlind *et al.*, 1988). In contrast, Fairbrother *et al.* (1989) demonstrated that STb$^+$ strains without any known adhesion factor could cause diarrhoea in experimentally inoculated newborn piglets.

In Hungary, Nagy *et al.* (1990) showed that 25% of the tested isolates produced one or more of the toxins STa_1 (also called STaP), STb and verotoxin (VT) without any known adhesins. Woodward & Wray (1990) similarly examined 1031 *E. coli* strains isolated from cattle and pigs with diarrhoea and observed that 24% of all toxigenic strains apparently did not possess adhesins. A possible explanation of the pathogenicity of STb$^+$ strains for which no adhesion factor can be detected is that these strains produce adhesion factors that are yet to be identified and characterized. For example, Kennan & Monckton (1990) described new adhesive fimbriae associated with porcine ETEC possessing STa and STb genes. An antiserum raised against the purified pilin did not cross-react with F_4, F_5 or F_6 fimbriae.

STb-induced colibacillosis seems to depend on the age of the animal. ETEC isolated from piglets with diarrhoea (333 isolates) at the age of 1–4 weeks produced one of the combinations of enterotoxins STb and STa (14·9%), STb and LT (52·1%) or STb alone (29·8%); piglets with diarrhoea at more than 4 weeks of age (412 isolates) produced the combinations STb and STa (20·4%), STb and LT (28·6%) or STb alone (33·4%). Most of the ETEC producing STb alone did not produce any of the known fimbrial antigens (J. M. Fairbrother, personal communication). Before 1 week of age, the number of strains producing only STb is lower (17%) (Moon *et al.*, 1986).

Thus, it seems that the STb gene is closely related to diarrhoea seen in pigs but can also be present in some ETEC strains isolated from healthy animals. The association of STb$^+$ strains with healthy animals could be explained by the absence of adhesion structures, non-susceptibility of individual animals, the absence of

predisposing environmental conditions or the production of minute amounts of toxin. In fact, *E. coli* strains can produce different amounts of STb toxin (Dubreuil *et al.*, 1991). The STb gene, although quite often found in association with the LT gene, is found predominantly as the single enterotoxin determinant in ETEC, which are isolated mostly from diarrhoeic animals.

Human strains

The presence of STb in ETEC strains of human origin was investigated to ascertain the animal species specificity of STb. Echeverria *et al.* (1984) reported the isolation of *E. coli* strains possessing the gene for STb from humans in Thailand. One STb^+ strain was isolated from a farmer without a recent history of diarrhoea. In a subsequent study, 1 ETEC strain hybridizing with the STb probe was found in 1 of 177 villagers with diarrhoea and in 12 of 1307 without diarrhoea (Echeverria *et al.*, 1985a). The STb^+ *E. coli* strain isolated from a woman with diarrhoea, and two strains isolated from villagers without diarrhoea, also contained the genes encoding LT. In this last study, STb^+ ETEC was shown to have been acquired from pigs.

Lortie *et al.* (1991c) reported that the gene encoding STb was detected in two *E. coli* strains isolated from the stools of humans with diarrhoea. Those two strains were also positive for cytolethal distending toxin (CLDT) and porcine heat-labile enterotoxin (LT1p). This study also demonstrated that, at least in those two human strains, the gene encoding STb was correctly expressed and that the enterotoxin produced was active in rat jejunal loops. However, these two strains lacked the classical fimbriae associated with animal ETEC. A causal relationship with diarrhoea could not be established in this case. ETEC strains isolated from children with diarrhoea under the age of 5 years in 4 tropical countries [Bangladesh (50 strains), Gambia (39), New Guinea (13) and Nicaragua (77)] were studied by Handl & Flock (1992). Only one of those isolates, originating from Nicaragua, was STb^+ and also produced LT and STa.

Okamoto *et al.* (1993) examined the production of STb by *E. coli* isolated from patients with traveller's diarrhoea. Of 400 strains examined, 3 produced STb. As in the study of Lortie *et al.* (1991c), they showed the STb gene sequence to be identical to that of porcine strains. The cultured *E. coli* strains induced fluid accumulation in ligated mouse intestinal loops. Fluid accumulation was neutralized by anti-STb antiserum to discriminate the effect of STb from that of LT1p which was also produced by these strains. Because fluid accumulation was noted in the absence of a protease inhibitor, these workers believed that their study strongly suggested that STb is occasionally a cause of human diarrhoea. Nevertheless, the role of STb in diarrhoea in humans remains to be determined. The low frequency of STb^+ human strains suggests that this enterotoxin could contribute, on rare occasions, to human diarrhoeal disease. STb^+ human ETEC isolates are most probably acquired from animals (e.g. pigs) and the role of STb in human diarrhoea has not yet been established directly. Thus, further studies are necessary to draw more definite conclusions about the enteropathogenicity of STb in humans.

Bovine strains

The STb gene is also found occasionally in ETEC strains isolated from cattle. Mainil *et al.* (1986) showed that 5 % of the *E. coli* strains isolated from calves carried the STb and LT genes. Another study by Mainil *et al.* (1990) of 870 ETEC strains isolated from cattle in Belgium revealed the absence of STb^+ strains. However, 7 of 12 bovine isolates obtained from the USA, tested in the same study, were positive for STb and for F_6 fimbriae. Work by Shin *et al.* (1994) indicated that 1·1 % of the 666 bovine isolates from the USA hybridized with the STb probe. Echeverria *et al.* (1985a) found one ETEC strain hybridizing with an STb probe in 162 water buffaloes. Thus, it seems that bovine ETEC strains carrying the STb gene are rare and none of the studies related the presence of the strains to diarrhoeal illnesses in the animal.

Chicken strains

Only one investigation (Akashi *et al.*, 1993) reported the isolation of *E. coli* strains containing the STb gene (7 of 38 strains) from the stools of chickens with diarrhoea. Culture supernatants from these strains induced fluid accumulation in the mouse intestinal loop test. When culture supernatants were tested in chicken intestinal loops, in the absence of the protease inhibitor required to obtain a positive response in mouse and rat loop assays, fluid accumulation was nevertheless observed. Production of STb at the chicken body temperature (41 °C) was observed and overall it was concluded that STb must play a role in chicken diarrhoeal diseases. No other studies on chicken ETEC isolates producing STb has been done to corroborate these data.

STb assays

Using the pig ligated intestine technique, Smith & Halls (1967) were the first to identify an *E. coli* cell-free heat-stable preparation that caused fluid accumulation. This demonstration of a heat-stable, enterotoxin-induced intestinal fluid response by culture supernatants of porcine diarrhoeagenic *E. coli* strains was confirmed by Smith & Gyles (1970) who coined the acronyms ST for stable toxin and LT for labile toxin.

As mentioned previously, Burgess *et al.* (1978) demonstrated that two distinct types of ST were elaborated by some *E. coli* strains. These heat-stable enterotoxins could be differentiated on the basis of methanol solubility, STb being precipitated by this organic solvent. Later it was found that the two enterotoxins could also be differentiated on the basis of their biological activity in different animal species (Whipp *et al.*, 1981; Weikel *et al.*, 1986c) leading to elaboration of *in vivo* assays in different animal species (Table 2).

Table 2. *In vivo* and *in vitro* STb assays

(a) ***In vivo*** **assays**

Animal species	Assay	Protease inhibitor	Reference
Pig	Intestinal loop	None	Smith & Halls (1967)
Calf, Lamb, Rabbit	Oral administration	None	
Pig	Intestinal loop (small intestine)	None	Smith & Gyles (1970)
Pig	Intestinal loop (jejunum)	Soybean trypsin inhibitor	Whipp (1987)
Pig	Intestinal loop (jejunum)	Soybean trypsin inhibitor	Whipp (1990)
Mouse (infant)	Oral administration	Soybean trypsin inhibitor	
Pig, Mouse, Rat, Rabbit, Calf	Intestinal loop (jejunum)	Soybean trypsin inhibitor	Whipp (1991)
Guinea-pig	Intestinal loop (ileum)	None	Choudhry *et al*. (1991)
Rat	Intestinal loop (jejunum)	Aprotinin	Fujii *et al*. (1991)
Mouse (infant)	Oral administration	Aprotinin	
Mouse	Intestinal loop (ileum)	None	Hitotsubashi *et al*. (1992a, b)
Mouse (infant)	Oral administration	None	
Chicken	Intestinal loop	None	Akashi *et al*. (1993)
Mouse	Intestinal loop	None	Fujii *et al*. (1995)

(b) ***In vitro*** **assays**

Assay	Reference
Radiolabelled DNA probe	Lee *et al*. (1983) Echeverria *et al*. (1984) Moon *et al*. (1986) Mainil *et al*. (1986, 1990) Monckton & Hasse (1988) Woodward & Wray (1990) Lortie *et al*. (1991a, b) Harel *et al*. (1991) Handl *et al*. (1992) Shin *et al*. (1994) Nagy *et al*. (1990)
Digoxigenin labelling of DNA	Lortie *et al*. (1991c) Boss *et al*. (1992) Riley & Caffrey (1990)
Alkaline phosphatase labelling	Medon *et al*. (1988)
PCR	Lortie *et al*. (1991c)
Direct ELISA	Urban *et al*. (1990a) Dubreuil *et al*. (1991) Lortie *et al*. (1991a) Lawrence *et al*. (1990)
Inhibition ELISA	Handl *et al*. (1988) Busque *et al*. (1995)
Immunoblot	Handl *et al*. (1988) Bossé *et al*. (1993)
Enhanced chemiluminescence immunodot	Lortie *et al*. (1991b)
Ussing chambers	Weikel *et al*. (1986c) Whipp *et al*. (1987) Hitotsubashi *et al*. (1992a)
Cell cultures	Dreyfus *et al*. (1993)

In vivo assays

For many years, the only *in vivo* assay for STb was the pig intestinal loop assay (Smith & Halls, 1967). The variability and inefficiency of this test for detection of the toxin was partly responsible for the fact that the STb toxin was only recently purified. In addition, this test was time-consuming and costly and could not be performed in every laboratory. Consequently, many epidemiological studies ignored the possible presence of STb.

It was not until Whipp (1987) showed that STb was susceptible to trypsin degradation and that variable amounts of trypsin-like activity in the swine jejunum were responsible for inconsistent responses to STb, that other animal species were used to set up more reproducible, less expensive *in vivo* assays. His study suggested that the concept of species specificity of STb toxin, which had been widely accepted at that time, should be re-examined. It now seems that failure to demonstrate a response to STb in species other than the pig could reflect the presence of one or more intraluminal factors, such as, for example, intrinsic protease activity, which precluded a response. A subsequent study (Whipp, 1990) showed that STb evoked a dose-dependent secretory response in infant mice and jejunal loops of rats as long as endogenous protease activity was blocked with soybean trypsin inhibitor. Variation in sensitivity to STb was noted for different animal species. Thus, infant mice were much less sensitive to STb than rats and pigs. A secretory response was also observed in rabbits and calves (Whipp, 1991). Other researchers also established that the guinea-pig intestine (ileal loop) was sensitive to STb (Choudhry *et al*., 1991).

In addition to trypsin inhibitor (soybean) used by Whipp (1987), aprotinin, a serine protease inhibitor, has been used by Fujii *et al*. (1991) in a suckling mouse assay and in a mouse intestinal loop assay (Fujii *et al*., 1995) to render animals susceptible to STb. Unexpectedly, a mouse intestinal loop assay (Hitotsubashi *et al*., 1992b) and a chicken intestinal loop assay (Akashi *et al*., 1993) were shown to demonstrate STb activity without addition of protease inhibitor. Thus, it seems that protease inhibitors are not always essential for the observation of a response to STb enterotoxin, depending on the experimental protocol used. In general, these results indicated that the receptor for STb, if required, and the mechanism by which STb stimulates intestinal secretion, are present and functional in the intestinal mucosa of a variety of animal species. Thus, STb cannot be categorized as a pig-specific enterotoxin.

In vitro assays

A variety of *in vitro* assays have now been developed to evaluate the presence or absence of the STb gene or protein, and in some cases even to measure the amount of enterotoxin produced (Table 2).

In general, radiolabelled specific DNA fragments obtained from enterotoxin genes can be used as probes to detect homologous sequences in *E. coli* isolates. As the sequence of the gene encoding STb has been determined, the gene encoding STb may be detected using a specific DNA probe. The first report of the use of such a probe was in a molecular epidemiological study (Lee *et al*., 1983). The study indicated that a DNA fragment containing the STb gene was effectively specific in detecting STb-producers among clinical *E. coli* isolates. The use of radioactive probes has frequently been used by researchers for STb determination (Echeverria *et al*., 1984; Moon *et al*., 1986; Mainil *et al*., 1986, 1990; Monckton & Hasse, 1988; Woodward & Wray, 1990; Lortie *et al*., 1991a, b; Harel *et al*., 1991; Handl *et al*., 1992; Shin *et al*., 1994; Nagy *et al*., 1990). In general, these assays possess the advantage of being very specific and sensitive.

Non-radioactive DNA labelling techniques for the detection of the STb gene include digoxigenin labelling of DNA (Lortie *et al*., 1991c; Boss *et al*., 1992; Riley & Caffrey, 1990) and alkaline phosphatase labelling (Medon *et al*., 1988). A simple PCR-based technique has also been developed (Lortie *et al*., 1991c).

In addition, antisera have been raised against STb, a poorly immunogenic molecule (Dubreuil *et al*., 1991), and a number of fusion proteins comprising STb. These antibodies have been evaluated for their capacity to detect specifically the STb enterotoxin in numerous immunological tests. Thus, direct ELISA (Urban *et al*., 1990a; Dubreuil *et al*., 1991; Lortie *et al*., 1991a; Lawrence *et al*., 1990) and inhibition ELISA tests (Handl *et al*., 1988; Busque *et al*., 1995), immunoblot assays (Handl *et al*., 1988; Bossé *et al*., 1993) and an enhanced chemiluminescence immunodot assay (Lortie *et al*., 1991b) have been developed. Overall, the specificity and sensitivity of these immunologically based assays depended on the polyclonal antibodies used. Anti-STb monoclonal antibodies were developed by Urban *et al*. (1991) using a protein A–STb fusion protein as the immunogen and an STb–alkaline phosphatase fusion protein to screen the clones. A total of four distinct anti-STb antibodies were obtained and characterized.

Intestinal membranes of animals have been used to measure a short-circuit current in Ussing chambers. Different research teams showed that porcine ileal tissues responded electrogenically to STb enterotoxin (Weikel *et al*., 1986c; Whipp *et al*., 1987). Interestingly, human ileal tissue showed no response to STb in this *in vitro* test, suggesting that the adult human ileum may lack the receptor for STb. Some authors also used intestinal membranes to rule out, for STb, known secretion mechanisms (Hitotsubashi *et al*., 1992a). Finally, cell cultures using cells of intestinal and non-intestinal origin were used by Dreyfus *et al*. (1993) to obtain information about the mechanism of action of STb.

Genetic aspects – the *estB* gene

Cloning and determination of the nucleotide sequence encoding STb was done independently (Lee *et al*., 1983; Picken *et al*., 1983). The gene encoding STb was referred

to as *estA* by different researchers (Mazaitis *et al*., 1981; Picken *et al*., 1983). This designation reflected the assumption that STb was one variant of STa enterotoxin. Today, this gene is referred to as *estB* since the nucleotide and amino acid sequences of STb bear no relationship to STa.

The *estB* gene that encodes STb is found on heterogeneous plasmids that may also determine other properties including other enterotoxins (i.e. LT, STa), colonization factors, drug resistance, colicin production and transfer functions (Harnett & Gyles, 1985; Echeverria *et al*., 1985b). *estB* has been shown to be part of a transposon of approximately 9 kb designated *Tn*4521 (Lee *et al*., 1985; Hu *et al*., 1987; Hu & Lee, 1988). This transposon is flanked by defective IS2 elements but is nevertheless functional as the STb gene can transpose from one plasmid to another (Lee *et al*., 1985; Hu & Lee, 1988). The structural gene for STb from different clinical isolates appears to be uniform in size but the flanking sequences are heterogeneous suggesting that *estB* could be found on different transposons (Lee *et al*., 1985). Thus it seems that transposition of *estB* is a mechanism by which this virulence factor is disseminated among ETEC.

The *estB* gene encodes a 71 amino acid polypeptide including a typical 23 amino acid signal peptide. Thus, mature STb polypeptide represents a 48 amino acid molecule. Final confirmation of the deduced amino acid sequence from the DNA came following purification and sequencing studies of the STb protein (Dreyfus *et al*., 1992).

Spandau & Lee (1987) indicated that the promoter for *estB* expression was weak. The promoter did not conform to the observed consensus sequence, as one important base in the Pribnow box (−10 region), the final invariant T, is replaced by a G. On the other hand, the −35 region is highly homologous to the −35 consensus sequence. Thus, the STb promoter is capable of binding RNA polymerase, but it is a poor initiator of transcription so very little STb is produced. Lawrence *et al*. (1990) indicated that cloning the *estB* gene into a high-expression vector downstream to the strong bacteriophage lambda p_L promoter increased by 10–20-fold the mRNA produced, but the amount of STb enterotoxin, as revealed by the bioassay, was not increased.

STb polypeptide

Biochemical characteristics

The STb polypeptide is synthesized as a 71 amino acid precursor (Lee *et al*., 1983, Picken *et al*., 1983). The NH_2-terminus of pro-STb (residues 1–23) has characteristics of a signal sequence that is cleaved, presumably by a signal peptidase, during export to the periplasm, and the periplasmic STb is then translocated to the extracellular medium (Kupersztoch *et al*., 1990). Mature STb is a 48 amino acid peptide containing 4 cysteine residues. Disulfide bridges exist between Cys-33 and Cys-71, and between Cys-44 and Cys-59 (Fujii *et al*., 1991) (Fig. 1). STb bears no homology to STa enterotoxin (Dreyfus *et al*., 1992). It loses biological activity upon β-mercaptoethanol or trypsin treatment (Dubreuil *et al*., 1991; Fujii *et al*., 1991). Out of 48 residues, the mature toxin contains only one tyrosine, two phenylalanine and no tryptophan residues, explaining the low absorbance at 280 nm (Handl *et al*., 1993). The determined isoelectric point of 9·6 corresponds to a highly basic protein (Handl *et al*., 1993). Purified STb is poorly immunogenic when injected into rabbits as numerous booster doses were required to obtain a significant antibody titre and the resulting polyclonal antiserum was non-neutralizing (Dubreuil *et al*., 1991). However, use of a fusion protein containing STb resulted in increased immunogenicity (Dubreuil *et al*., 1996) and neutralizing antisera were raised.

Production characteristics

STb is not associated with the cellular fraction but found preferentially in the culture supernatant (Kupersztoch *et al*., 1990). Many studies have reported poor yield of toxin from wild-type strains (Urban *et al*., 1990b; Dubreuil *et al*., 1991). The production of STb by wild-type *E. coli* strains was recently studied using a quantitative STb-specific inhibition ELISA (Busque *et al*., 1995). Variation was found between the wild-type strains and the production also varied with the liquid culture medium used. Thus, STb production is controlled by growth conditions. A repressive effect of glucose on STb production and a reversal of this effect upon addition of cAMP was observed. Catabolite repression of STb was confirmed using mutant strains for adenylate cyclase and catabolite activator protein. A DNA homology search revealed a sequence with 72% identity with the cAMP receptor protein (CRP)-binding site located 26 bp upstream of the −35 region of the transcriptional start site of *estB*.

Purification of STb

Purification of STb, as stated before, has been hampered due to the low levels of toxin produced by wild-type *E. coli* strains and the lack of a convenient *in vitro* assay. Nevertheless, native STb has been purified from a wild-type strain (Dubreuil *et al*., 1991) and from strains carrying a recombinant plasmid (Fujii *et al*., 1991; Dreyfus *et al*., 1992). Studies describing purification from a cloned hybrid fusion protein, where STb could be cleaved and purified to homogeneity, were reported by Bossé *et al*. (1993) and Handl *et al*. (1993).

Secretion and disulfide bond formation

For conversion into active STb, the intramolecular bonds must be correctly formed. The pathways by which these bonds are formed have been established. Kupersztoch *et al*. (1990) showed that STb is a 48 amino acid molecule corresponding to a 5·2 kDa extracellular

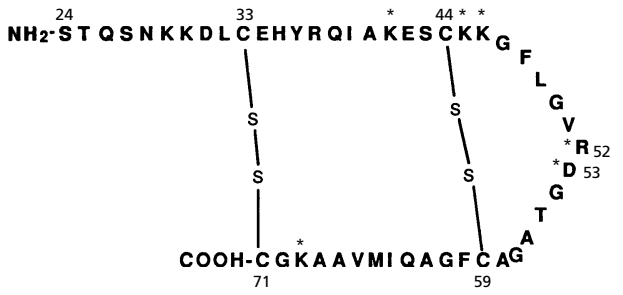

Fig. 1. Amino acid sequence of mature STb. The sequence is numbered taking into account the first 23 amino acids residues representing the signal sequence. Note the position of the disulfide bridges between Cys-33 and Cys-71, and Cys-44 and Cys-59. Asterisks indicate residues shown to be important for STb toxicity.

polypeptide. This molecule is a transient periplasmic species and a single processing event on a precursor results in it becoming extracellular. Thus, an 8·1 kDa precursor (pre-STb) is converted to a transiently cell-associated 5·2 kDa form. After STb is detected as a cell-associated molecule, an indistinguishable extracellular form becomes apparent, suggesting that no proteolytic processing occurs during mobilization of STb from the periplasm to the culture supernatant. Conversion of cellular to extracellular STb does not depend on membrane potential or oxidative phosphorylation. Conversion of pre-STb to cellular STb depends on the *secA* gene product. For STb, like STa, translocation of the precursor to the periplasm requires energy (Kupersztoch *et al*., 1990). These data indicate that export of STb relies on the general export pathway of *E. coli*.

Foreman *et al*. (1995) obtained secretion-deficient mutants using a synthetic transposon. In *dsbA* and *tolC* mutants, STb was absent from the culture supernatant indicating that these genes were required for secretion of STb. Mutations in *tolC* and *dsbA* produced a STb^- phenotype because the precursors did not cross the outer membrane after they had reached the periplasm; instead they were periplasmically degraded.

Concerning disulfide bond formation, Dreyfus *et al*. (1992) explored the role of the four cysteine residues in STb secretion. Cys-44 and Cys-71 were separately substituted with serine residues. The resulting peptides were exported to and degraded in the periplasm, suggesting that formation of disulfide bridges protected STb, a lysine (6) and arginine (2) containing peptide, from a trypsin-like activity. Therefore, as observed by Foreman *et al*. (1995), a *dsbA* mutant forming disulfide bridges at a slower rate yielded a STb^- phenotype as the reduced form was degraded. Similarly, Okamoto *et al*. (1995) transformed a *dsbA* mutant with a plasmid harbouring *estB*; STb was not detected either in the cells or in the culture supernatant. In this study, STb production was shown to be restored by introducing the wild-type *dsbA* gene into the mutant strain. Thus, it was confirmed that *dsbA* is involved in forming the disulfide bonds in STb and that its absence resulted in degradation of STb during secretion. Using oligonucleotide-directed site-specific mutagenesis of the four cysteine residues it was established by Arriaga *et al*. (1995) that the two intramolecular disulfide bonds must be formed for the efficient secretion of STb. Elimination of either one of the bonds renders the toxin susceptible to periplasmic proteolysis and abolishes its toxicity.

Residues related to toxicity and structure determination

As the isoelectric point of 9·6 indicates, the side-chain of some of the basic amino acid residues project outside the STb molecule. Some studies have been conducted to determine the role of selected amino acids, and in particular of the basic residues, on the toxicity of STb. Among the 48 amino acids composing STb, 9 are basic (1 His, 2 Arg and 6 Lys).

A loop defined by the disulfide bond between Cys-44 and Cys-59 containing 14 amino acids (Fig. 1), including four Gly residues and four of the charged residues based on secondary structure predictions, suggested the presence of an extended coil region (Dreyfus *et al*., 1992). The location of a Arg-52 and Asp-53 charged pair inside this loop is such that it could be highly exposed in a hydrophilic environment and the authors speculated that it would probably be involved in receptor recognition. Using site-directed mutagenesis, substitutions were performed on these two residues. When Arg-52 was changed to Ser to eliminate the charge, a significant reduction in specific activity of the mutant molecule was noted, a smaller reduction of toxicity was associated with the substitution of Asp-53. No reduction in stability of the altered molecule was noted in the intestinal loop model. The mutated toxins (Ser replacing the charged amino acids) did not interfere with the toxic activity of native STb, suggesting that they were not competing for the putative STb receptor.

Fujii *et al*. (1994) likewise investigated the role of basic amino acid residues on STb enterotoxicity. Studies involving chemically modified STb indicated that Lys residues play an important role in STb toxicity and that the contribution of other basic residues to toxicity is relatively low. These results were confirmed using oligonucleotide-directed mutagenesis. When Lys residues at positions 41, 45, 46 and 69 were replaced by neutral amino acids, the toxicity of the molecule was reduced. Changes of Lys-45 and Lys-46 resulted in a considerable reduction in toxicity.

Sukumar *et al*. (1995) determined the solution structure of STb by two- and three-dimensional NMR methods. The NMR-derived structure showed that STb is helical between residues 33 and 46 and residues 61 and 67. The helical structure in the region 33–46 is amphipathic and exposes several polar residues to the solvent, some of which have been shown to be important in toxicity. The loop region between residues 44 and 59 contains a cluster of hydrophobic residues and exposes Arg-52 and Asp-53. These residues, as discussed before, are important in toxicity. Circular dichroism studies indicated

that the integrity of the disulfide bridges is crucial for the structure and function of the toxin as reduced STb adopted a random coiled conformation. STb is thus a highly organized molecule with $73 \pm 2\%$ helix, $4 \pm 2\%$ β-structure and 22% remainder. Overall, the results indicated that the peptide spanning the region from Cys-33 to Cys-71 has full activity and that the peptide consisting of the first 7 amino acids at the NH_2-terminus is not involved in either the structure or toxicity of STb. This characteristic was also observed for STa (Gariépy *et al*., 1986).

Mechanism of action

Initial studies to determine the mechanism of action of STb were performed with crude culture filtrates of STb^+ ETEC strains. Kennedy *et al*. (1984) first reported that STb induced fluid secretion after 3–6 h and that the toxin did not disrupt intestinal histology. STb stimulated cyclic-nucleotide-independent secretion. Thus, STb appeared as a cytotonic toxin with properties and a mechanism of action differing from STa. Using Ussing chambers and crude culture filtrate containing STb, Weikel & Guerrant (1985) showed that the toxin promptly increased the short circuit current and potential difference. The toxin did not alter unidirectional or net fluxes of ^{22}Na or ^{36}Cl when tested in bidirectional *in vitro* studies. Relative to controls, significant accumulation of Na and Cl occurred intraluminally *in vivo*. Measurements of the electrolyte content of ligated intestinal segments *in vivo* further suggested that STb stimulated bicarbonate secretion (Fig. 2).

Whipp *et al*. (1985) observed that a virus-induced loss of villous epithelium resulted in an absence of response to STb. The secretory response of STb thus seemed to be dependent on the integrity of the villous epithelium. Contradictory observations were reported in different studies. For example, Whipp *et al*. (1986) observed that exposure of swine jejunum to a culture supernatant containing STb induced microscopic alterations of intestinal mucosa, in particular the loss of villous absorptive cells and partial atrophy of villi, which are consistent with the loss of absorptive capacity. The toxin was capable of causing partial villous atrophy in pigs after only 2 h (Whipp *et al*., 1987; Rose *et al*., 1987). Using light microscopy, Hitotsubashi *et al*. (1992b) observed that exposure of the mouse jejunum to purified STb for 3 h caused a dilation of capillaries of the submucosa and a decrease in the thickness of the lamina propria. No cellular damage or inflammation was observed. Thus, it appeared that damage to the epithelium occurred when culture supernatant containing STb, but not the purified toxin, was used.

Using a mouse intestinal loop assay and purified toxin, Hitotsubashi *et al*. (1992b) confirmed that STb did not alter cGMP or cAMP levels in intestinal mucosal cells, thus indicating that the mechanism of action of STb in inducing fluid secretion differs from that of STa and cholera toxin (CT). The level of prostaglandin E_2 (PGE_2) in the fluid increased as a result of STb action and prostaglandin synthesis inhibitors such as aspirin and indomethacin significantly reduced the response to STb. This report was the first to implicate PGE_2 in the mechanism of action of STb. CT was previously shown to increase PGE_1 and PGE_2 contents in the intestinal tissues of rabbits, indicating that prostaglandins may regulate water and electrolytes in cholera (Peterson & Ochoa, 1989). A histopathological study indicated that STb increased the blood volume resulting in dilation of capillaries. Thus, PGE_2 levels are involved in the onset of diarrhoea in intestinal mucosal cells. Although intraluminally administered PGE_2 is known to induce duodenal and jejunal secretion of water and of electrolytes such as Cl^- and Na^+ (Rask-Madsen & Bukhave, 1981), the precise mechanism by which these secretions are induced is not yet known.

More recently, Fujii *et al*. (1995) confirmed that the quantity of PGE_2 produced by intestinal cells was directly related to the dose of STb administered to the mouse. In addition, the quantity of PGE_2 correlated with the volume of fluid released into the intestinal lumen. Levels of arachidonic acid and phosphatidic acid were also elevated by STb, indicating that arachidonic acid metabolism is stimulated by STb.

Two groups have independently reported that both PGE_2 and 5-hydroxytryptamine (5-HT), the latter being regarded as another secretagogue, were released into the intestinal fluid (Harville & Dreyfus, 1995; Peterson & Whipp, 1995). Peterson & Whipp (1995) compared the secretory effects of CT, STa and STb using the pig intestinal loop model and measured the effects of those toxins on the synthesis of cAMP, cGMP and PGE_2 as well as the release of 5-HT from intestinal enterochromaffin cells. A combination of maximal doses of STa and STb yielded additive effects on fluid accumulation, suggesting different mechanisms of action. A similar additive effect on fluid accumulation and luminal release of 5-HT was noted with a combination of CT and STb. A cAMP response to STb could not be demonstrated in either mucosal tissue or luminal fluid. Thus, it appears that the mechanisms of action of STb and CT are distinct. Treatment of rats with ketanserin, a 5-HT receptor antagonist, reduced intestinal secretion induced by STb (Harville & Dreyfus, 1995). Nonetheless, the mode of action of STb may be similar to that of CT since the latter toxin stimulates the release of both PGE_2 and 5-HT, suggesting a potential effect on the enteric nervous system. Interestingly, a study by Eklund *et al*. (1985) using an *E. coli* strain producing both STa and STb showed that, at least in rats and cats, these heat-stable enterotoxins evoke secretion in part via activation of the enteric nervous system. Drugs influencing nervous activity, such as lidocaine, tetrodotoxin and atropine, significantly diminished the secretory response. Furthermore, a study using isolated mouse ileum indicated that STb could also act directly on the muscle cells of the ileal serosa increasing the spontaneous motility of the intestine and resulting in contractions (Hitotsubashi *et al*., 1992a). These contractions were not induced when the toxin was applied to

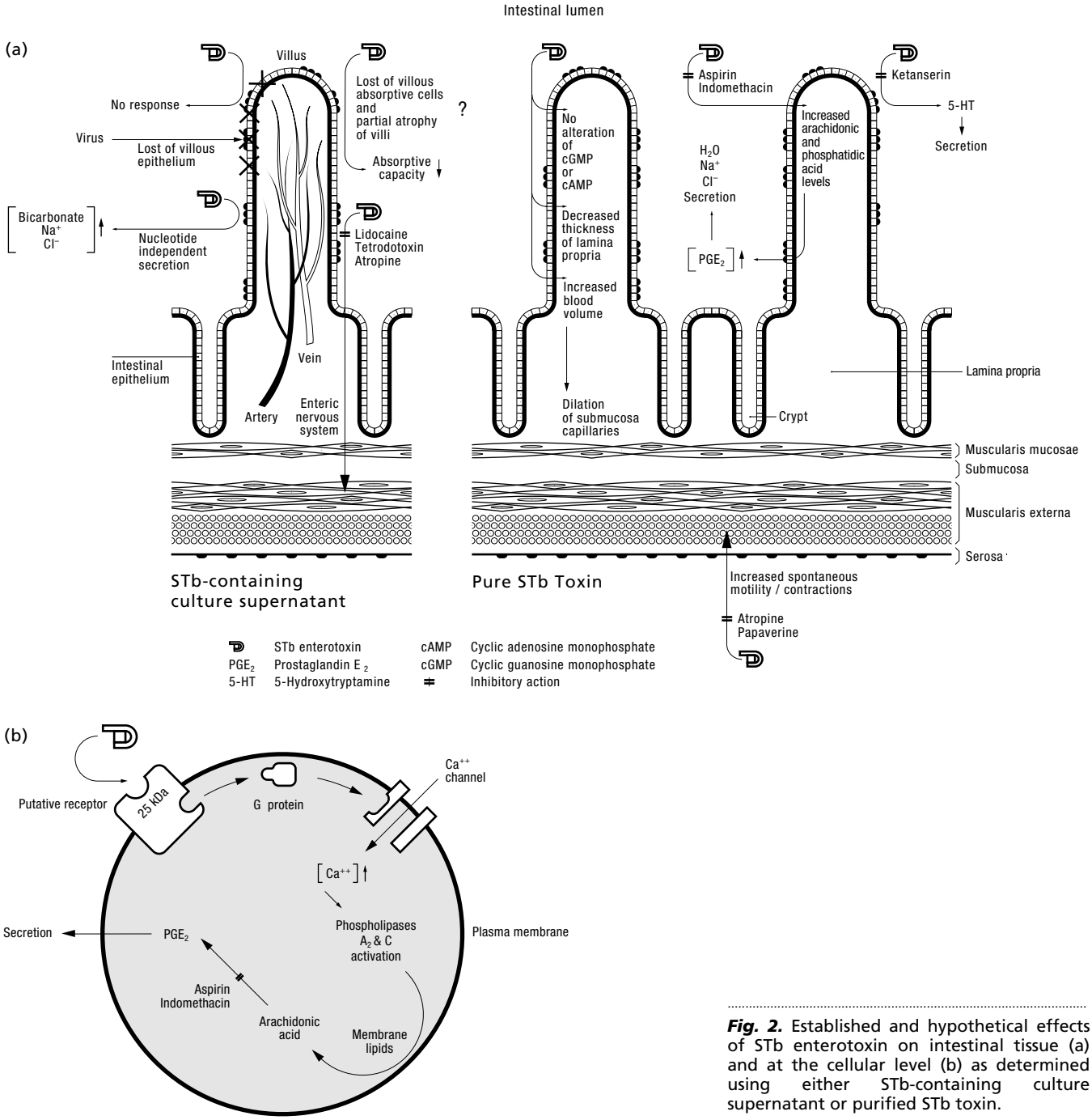

Fig. 2. Established and hypothetical effects of STb enterotoxin on intestinal tissue (a) and at the cellular level (b) as determined using either STb-containing culture supernatant or purified STb toxin.

the mucosa. The spontaneous motility was inhibited by atropine, indicating that it was not the result of the excitation of cholinergic nerves. Papaverine, which causes relaxation of smooth muscle, had an inhibitory effect on STb, implying that STb acts directly on muscle cells.

An *in vitro* study suggested that STb functions by opening a G-protein-linked, receptor-operated calcium channel in the plasma membrane (Dreyfus *et al*., 1993). A dose-dependent increase in intracellular Ca^{2+} that was linked to extracellular Ca^{2+} was observed. This process could be blocked by agents that impair GTP-binding regulatory function. Elevated intracellular Ca^{2+} activates both phospholipase A2 and phospholipase C, two important enzymes for the release of arachidonic acid from membrane lipids (Dunlop & Larkins, 1984). It thus appears that the initial action of STb in the induction of diarrhoea is the uptake of Ca^{2+} into cells. Subsequently, synthesis of PGE_2 and other secretagogues is stimulated, leading to the induction of diarrhoea.

Receptor identification

Hitotsubashi *et al*. (1994) identified a protein of 25 kDa from the cell membranes of mouse intestine that binds to

STb. The specificity of the interaction was corroborated by competition experiments between radiolabelled (^{125}I) and unlabelled toxin. Preliminary characterization of the molecule suggested that it was not a glycoprotein as it did not bind to concanavalin A–Sepharose nor was it digested with endo-β-glycosidase H as observed by SDS-PAGE. This study also indicated tissue specificity as the STb-binding protein was not found in mouse tissues such as liver, lung, spleen and kidney. No lesions were evident in any organs following visual inspection after STb has been administered intravenously at 1 mg per mouse. Surprisingly, Dreyfus *et al*. (1993) inferred from an *in vitro* study that the STb receptor is present on cell types of both intestinal and non-intestinal origin, as Madin-Darby canine kidney, HT-29/C_1 intestinal epithelial cells and primary rat pituitary cells all responded to STb. In all these cell types, a dose-dependent increase in calcium concentration due to STb action was noted.

A study by Weikel *et al*. (1986b) examining the response in a Ussing chamber of human adult ileal mucosa to STb clearly indicated that, in contrast to the piglet jejunum which responded electrogenically to STb, human tissue showed no response. From their work, they inferred that the adult human ileum could lack the receptor for STb. With the present state of knowledge, it is difficult to understand the discrepencies between the different studies on the STb receptor.

Perspective

The recent purification of STb enterotoxin has helped to clarify some biochemical and mechanistic aspects of this toxin. Mutagenesis experiments have contributed to defining reactive sites on the polypeptide and three-dimensional structure determination will be needed to understand more fully the toxin–receptor interaction when the receptor has been definitively identified. A more thorough investigation of the mechanism of action of STb is crucial for our understanding of the pathogenesis of diarrhoeal illness and to devise effective therapeutic strategies which could interfere with the secretory pathways.

Finally, the study of the STb toxin may have suffered from being originally considered as a pig-specific toxin. However, now that at least some human *E. coli* strains producing STb have been isolated, although not directly related to diarrhoea in man, a new interest in this enterotoxin may be generated in the coming years.

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