Fusion of the genes encoding *Escherichia coli* heat-stable enterotoxin b (STb) and the maltose-binding protein to obtain mature STb enterotoxin

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The heat-stable enterotoxin b gene (*estB*) of *Escherichia coli* was fused to the gene for maltose-binding protein (*malE*). The *estB* gene was cloned into the pMAL-p vector using PCR. The construct consists of the signal sequence of maltose-binding protein, which directs the export of the fusion protein to the periplasm, and the maltose-binding protein fused to the STb polypeptide. A sequence encoding a factor Xa cleavage site is present between *malE* and *estB*. The fused genes are controlled by P_{tac}, a strong inducible promoter. Following IPTG induction, the recombinant strain expressed a 47 kDa protein, which was easily purified from osmotic shock fluid by using preparative electrophoresis and electroelution. Cleavage of the fusion protein with factor Xa generated the maltose-binding protein (42 kDa) and a polypeptide of approximately 5 kDa, corresponding to the molecular mass of mature STb. A monospecific polyclonal rabbit antiserum raised against purified STb reacted in immunoblot with the fusion protein and the cleaved-off peptide. A positive response was observed when testing the osmotic shock fluid containing the fusion protein in a rat intestinal loop assay. On average, 3–4 mg of MBP-STb protein was recovered per litre of induced recombinant strain.

Introduction

To date, enterotoxigenic *Escherichia coli* (ETEC) strains of porcine origin have been reported to produce two types of proteinaceous heat-stable enterotoxins. STa is methanol-soluble and induces intestinal secretion in infant mice and neonatal pigs, whereas STb is methanol-insoluble and induces intestinal secretion in weaned and neonatal pigs but does not affect infant mice (Burgess *et al.*, 1978; Weikel *et al.*, 1986). Despite similarities in heat-stability and association with transposons, STa and STb are distinct toxins (So *et al.*, 1979; So & McCarthy, 1980; Lee *et al.*, 1983; Hu *et al.*, 1987). STb appears to be a significant contributor to swine diarrhoea; the *estB* gene is the toxin gene most commonly found in association with ETEC of diseased swine (Mainil *et al.*, 1986; Moon *et al.*, 1986; Harel *et al.*, 1991; Handl *et al.*, 1992). In addition, diarrhoea has been induced in specific pathogen-free piglets by administration of *E. coli* strains

producing STb toxin alone (Picken *et al.*, 1983; Fairbrother *et al.*, 1989). The heat-stable enterotoxins reversibly alter normal intestinal homeostasis (Sack, 1975), causing intestinal secretion and diarrhoea (Betley *et al.*, 1986; Weikel *et al.*, 1986).

Research on the mode of action of STb toxin has been hampered due to a low level of toxin production by wild-type strains and lack of a convenient *in vitro* assay. Purification of the native toxin by our group allowed development of convenient and rapid assays for STb enterotoxin (Dubreuil *et al.*, 1991; Lortie *et al.*, 1991*a, b*). In fact, one test was sensitive enough to detect the toxin in stools of a specific pathogen free piglet infected with an STb-positive *E. coli* strain (Lortie *et al.*, 1991*c*). Recently, Fujii *et al.* (1991) reported the purification of STb by ammonium sulphate fractionation and successive column chromatographies from the culture supernatant of a strain harbouring the plasmid encoding the *estB* gene.

The cloned and sequenced *estB* gene (Lee *et al.*, 1983; Picken *et al.*, 1983), encodes a 71 amino acid polypeptide, including a 23 amino acid signal sequence (Kupersztoch *et al.*, 1990). This toxin is found in the bacterial periplasm

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as an 8.1 kDa precursor that is converted to and secreted as a 5.2 kDa mature protein (Kupersztoch *et al.*, 1990; Dubreuil *et al.*, 1991; Fujii *et al.*, 1991). The amino acid sequence determined by Kupersztoch *et al.* (1990) coincides fully with the 48 carboxy-terminal amino acids inferred from the DNA sequence. In the same study, serine at position 24, was shown to be the amino terminus of mature STb. Spandau & Lee (1987) suggested that *estB* gene was regulated by a weak promoter, explaining why very little STb toxin is produced by wild-type strains.

Numerous studies have described genetic fusions with the *estB* gene (Spandau & Lee, 1987; Handl *et al.*, 1988; Lawrence *et al.*, 1990; Urban *et al.*, 1990a, b). However, these fusions could not serve as a source of free and biologically active STb enterotoxin. Although STb could be purified from culture supernatants of a wild type strain and a strain containing a recombinant plasmid, these methods did not provide a convenient source of readily obtainable mature STb enterotoxin (Dubreuil *et al.*, 1991; Fujii *et al.*, 1991).

In this study, we describe a genetic construct between *malE* gene, coding for a maltose-binding protein (MBP), and *estB*. This genetic construct is under the control of the strong promoter P_{tac} and can thus be induced. Mature STb molecule was obtained after specific cleavage of the fusion protein by using an Xa protease. Expression of the fusion protein and its biological properties were also analysed. The MBP-STb hybrid protein is a readily obtainable source of active STb enterotoxin.

Methods

Strains and plasmids. The recombinant plasmid pRAS1 (Lee *et al.*, 1983) was used to amplify the *estB* gene. Plasmid constructs were cloned into strain HB101. The vector pMAL-p was purchased from New England Biolabs. The pMAL-p plasmid harbours a gene (*malE*) coding for maltose-binding protein (MBP) controlled by a strong inducible promoter P_{tac} (Duplay *et al.*, 1984; Maina *et al.*, 1988). A polycloning site permits subcloning of a foreign gene at the 3' end of *malE*. A sequence specific for factor Xa (a proteolytic enzyme) is present between *malE* and the fused gene.

DNA manipulations. Plasmid isolation was done by using the alkaline lysis method and plasmid DNA was purified by using ultracentrifugation in a caesium chloride gradient (Maniatis *et al.*, 1982). The restriction enzymes *Bgl*II, *Hind*III, *Sma*I, *Xba*I, T4 ligase, and mung bean nuclease were purchased from Pharmacia and used according to the manufacturer's recommendations. The pMAL-STb DNA construct was sequenced using the T7 Sequencing kit (Pharmacia). The *estB* PCR product used as a DNA probe was purified on acrylamide gel, eluted as described by Maniatis *et al.* (1982) and labelled by the random priming method using nanomers and T7 DNA polymerase (T7 Quickprime labelling kit, Pharmacia). Clones were screened by colony hybridization using an *estB* PCR product. DNA hybridization was done according to the method of Lortie *et al.* (1991a). The radioactive isotopes used

were [α - 32 P]dCTP [3000 Ci (111 TBq) mmol⁻¹] and [α - 35 S]dATP [500 Ci (18.5 TBq) mmol⁻¹] (New England Nuclear Research). Electrotransformations of recombinant plasmids were done with a Gene Pulser (BioRad) following the manufacturer's protocol.

Polymerase chain reaction (PCR) for subcloning. The DNA sequence coding for the mature STb protein was amplified enzymically. Oligonucleotides were designed with specific restriction site sequences to allow unidirectional subcloning into pMAL-p. The restriction sites *Xba*I and *Hind*III were added to the 5' and 3' ends of the *estB* gene, respectively. The *Xba*I restriction site sequence was added at the 5' end of the oligonucleotide SXF (5'GCCTCTAGAGTACACAATCAAA-TAAAAA3') that hybridized specifically to the nucleotides 516–532 of *estB* according to Lee *et al.* (1983). The oligonucleotide SHR (5'TGTATAAGCTTCTGAATGCTATTG3') hybridized specifically to nucleotides 669–693 and the SHR oligonucleotide included nucleotide mismatches in order to create an *Hind*III restriction site. Amplification, at low stringency, was performed as described previously (Lortie *et al.*, 1991a) with the following changes: 5 cycles of denaturation steps at 94 °C for 30 s, an annealing step at 40 °C for 30 s, and a synthesis step at 72 °C for 1 min. Twenty-five cycles were done at higher stringency using the following cycle: 94 °C for 30 s, 60 °C for 30 s, 70 °C for 1 min. The reaction was completed at 72 °C for 7 min. The reaction mix contained 50 mM-KCl, 1.5 mM-MgCl₂, 10 mM-Tris/HCl (pH 8.3), 12.5 µM-NTPs, 100 pmol SXF and SHR respectively, 0.3 pmol pRAS target DNA and 0.5 units *Taq* polymerase in 100 µl volume. After amplification, the DNA was immediately phenol/chloroform-extracted and ethanol-precipitated. The DNA was thereafter resuspended in 20 µl 10 mM-Tris/HCl (pH 8.0) containing 1 mM-EDTA. Oligonucleotides were synthesized by Bio-Synthesis. The enzymic DNA amplification was performed on a DNA Thermal Cycler model 480 using the Geneamp kit (Perkin Elmer Cetus).

Immunocolony screening. Clones were inoculated on Luria Broth agar medium containing 50 µg ampicillin ml⁻¹ and 0.3 mM-IPTG (Sigma) and incubated for 4 h at 37 °C. Cells were then lysed by chloroform treatment and transferred onto cellulose filters (Schleicher & Schuell). The conditions used for immunological reactions were identical to the Western blot protocol described below. pMAL-STb clones were screened immunologically with anti-STb antibodies (Dubreuil *et al.*, 1991).

Electrophoresis. SDS-PAGE was done according to Laemmli (1970) in a Mini-slab apparatus (BioRad). Total cell extract or purified protein solubilized in sample buffer was stacked in a 4.5% (w/v) acrylamide gel (100 V, constant voltage) and separated on a 10% (w/v) acrylamide gel (200 V). Low molecular mass proteins were analysed using the PhastSystem (Pharmacia) and high density SDS-PAGE PhastGel system according to the manufacturer's instructions. Proteins were stained with Coomassie Blue R-250. Gels were scanned using a LKB Ultrosan XL enhancer laser densitometer (LKB) to evaluate the relative amount of the fusion protein present in the osmotic shock fluid (OSF).

Immunoblotting. When required, proteins were transferred from slab gels to nitrocellulose paper using the methanol/Tris/glycine system described by Towbin *et al.* (1979). Electroblothing was done in a Hoefer transblot apparatus for 45 min at 100 V. All incubations were at room temperature unless otherwise stated. Membranes were blocked with 20 mM-Tris/HCl (pH 7.5), 0.5 M-NaCl (TBS) to which 9% (w/v) skim milk was added. Primary antibodies were incubated for 12–16 h at 4 °C. The polyclonal rabbit anti-STb antibodies were diluted 1:100 and the polyclonal anti-MBP antibodies 1:10000 in TBS containing 2% (w/v) skim milk. Peroxidase-conjugated goat anti-rabbit IgG (H + L) (Jackson ImmunoResearch) diluted 1:500 in TBS containing 2% (w/v) skim milk was incubated for 1 h at 4 °C. Reactive bands were visualized

by the addition of 0.3% 4-chloro-1-naphthol (Sigma) in cold methanol and 0.06% H₂O₂ in Tris/saline (Hawkes, 1982). Prestained SDS-PAGE low range markers (BioRad) and peptide molecular mass markers (Pharmacia) were used.

For detection of STb peptide, Immunodyne membrane (Pall Biosupport Division) was precut to convenient sizes and layered on top of prerun PhastGels using the PhastSystem (Pharmacia). Protein transfer was done by passive diffusion at 65 °C by overlaying the membrane with a pile of filter paper and a heavy weight. After transferring for 30 min the membrane was blocked for 30 min in 5% (w/v) skim milk in phosphate-buffered saline (PBS; 10 mM, pH 7.4). Rabbit sera against protein A-STb fusion (Handl *et al.*, 1988) was added to the blocking solution (1:500) and incubated for 2 h. Membranes were washed 3 times for 5 min with PBST (PBS containing 0.1% Tween 20), incubated for 1 h with alkaline-phosphatase-conjugated goat anti-rabbit IgG antibodies (Sigma) (1:4000), washed 3 times for 5 min with PBST and 2 min with H₂O and thereafter developed. The reactive bands were visualized as described by Blake *et al.* (1984) with 5-bromo-4-chloro-3-indolyl phosphate (Boehringer) as the alkaline phosphate substrate and nitro blue tetrazolium (Boehringer) as the colour development reagent.

Immunodotting. A Bio-Dot microfiltration apparatus (BioRad) was used according to the manufacturer's protocol for immunodot analysis of samples. Samples were applied in the wells to the Immunodyne membrane, incubated for 30 min and then carefully sucked out. After rinsing wells with 200 µl PBS the membrane was dried and immunostained according to above description for STb immunoblotting.

Purification of the fusion protein. An 18 h culture of the pMAL-STb clone was diluted 100 times in 1 litre rich medium (per litre: 10 g tryptone, 5 g yeast extract, 5 g NaCl and 50 µg ampicillin ml⁻¹) and kept at 37 °C. To induce expression of the fusion protein, IPTG was added to a final concentration of 0.3 mM when the OD₆₀₀ reached 0.4. Three hours after induction, cells were harvested by centrifugation at 4000 g for 10 min at 4 °C. Cells were gently washed with 250 ml 30 mM-Tris/HCl (pH 8.0), containing 20% (w/v) sucrose and 1 mM-EDTA. After centrifugation at 8000 g for 10 min at 4 °C, cells were suspended in 250 ml 5 mM-MgSO₄ at 4 °C for 10 min. Following further centrifugation at 8000 g for 10 min at 4 °C, the supernatant representing the osmotic shock fluid (OSF) was filter sterilized (0.20 µm) and lyophilized.

Lyophilized OSF was reconstituted in sample buffer (Laemmli, 1970) to 10 times the original concentration, loaded on to 12.5% (w/v) polyacrylamide gels (12 × 10 × 0.22 cm) and run at 100 V for 45 min followed by 200 V for 3.5 h. After migration, a small part of the gel containing the sample and the molecular mass markers was Coomassie-stained. The protein band at 47 kDa was excised, minced, and electroeluted using an electroeluter Model 422 (BioRad). The electroelution was performed at 25 mA per glass tube for 6 h at 4 °C in 50 mM-NH₄HCO₃ buffer using membrane caps with a 14 kDa cut-off. Electroeluted material was dried by using a Speedvac evaporator. The fusion protein was desalted on a Fast Desalting Column HR 10/10 (FPLC Systems, Pharmacia) and fractions were analysed for STb by immunodot. The exclusion limit of the column was 5 kDa. The column was equilibrated with Xa enzyme buffer (20 mM-Tris/HCl (pH 8.0), 100 mM-NaCl, 2 mM-CaCl₂, 1 mM-Na₃N₃) diluted 20 times. The eluted material was then concentrated 20 times. Twenty microlitres (0.3 mg ml⁻¹) of recovered MBP-STb was cleaved with 1 µl containing 1 U of Xa enzyme (New England BioLabs) for 3 h at room temperature. Samples were analysed on high density SDS-PAGE PhastGel.

Rat intestinal loop assay. The rat intestinal loop assay was performed as described previously (Dubreuil *et al.*, 1991). Briefly, 6-to-8-week-old white rats (Sprague Dawley, C.D.) were used. The animals were fasted for 48 h, anesthetized, and the abdomen was cut following a midline

incision. The small intestine of the rat was located. A volume of 8 ml of 20 mM-Tris/HCl (pH 6.8) plus 300 µg soybean trypsin inhibitor (Boehringer) was injected into the small intestine and left for 5 min. The liquid was then massaged and removed through the posterior end of the small intestine. A total of 8 loops (5 cm in length) were made and 0.5 ml of sample was injected into each loop. After 4 h of incubation, the rat was killed, and the volume (ml) and the length (cm) of each loop were measured. Results were expressed as volume of liquid (ml) per length (cm) × circumference (cm) of intestine and were considered positive if greater than 0.05. Each sample was tested at least 4 times in loops at different positions of the small intestine.

Results

This study involved the construction of hybrid plasmids to overexpress *Escherichia coli* heat-stable enterotoxin type b (STb). A sequence coding for a Xa cleavage site was located at the N-terminus of the mature STb protein. Specific cleavage of the maltose-binding protein (MBP)-STb fusion molecule with the Xa enzyme generated mature STb toxin. A two-step strategy was used to clone *estB* into pMAL-p. An intermediate clone pMAL-IN, where *estB* is not in the same translational reading frame as the vector *malE* gene, was first generated. In the final construct pMAL-STb, which was derived from pMAL-IN, the same translational reading frame was established for both *estB* and *malE*.

The *estB* gene obtained from plasmid pRAS1 was inserted into the pMAL-p vector by using PCR. The PCR product was subcloned into the *Xba*I and *Hind*III polylinker restriction sites of the vector (Fig. 1). Clones were screened by colony hybridization using the *estB* PCR product as a probe. The intermediate clone pMAL-IN did not express mature STb, since *estB* was subcloned out of frame with *malE*. pMAL-IN was characterized by using the restriction enzymes *Xba*I, *Hind*III and *Bgl*III. *Bgl*III digestion of pMAL-IN generated an 800 bp fragment corresponding to the *Bgl*III restriction sites in the *malE* gene and the inserted *estB* PCR product. The pMAL-IN clone produced a 45.2 kDa fusion protein consisting of MBP (42 kDa) and a 3.2 kDa peptide. Strain HB101 harbouring pMAL-IN reacted with anti-MBP polyclonal antibodies in immunoblot (Fig. 2b, lane 4). This polypeptide was coded by a sequence starting at the *Xba*I site of the polylinker, and the *estB* gene was read out of frame until a stop codon was reached. The fusion protein produced by pMAL-IN did not react in immunoblot with anti-STb rabbit polyclonal antibodies (Fig. 2a, lane 4).

The final construct, named pMAL-STb, was derived from the intermediate clone pMAL-IN (Fig. 1). In order to create a MBP-STb fusion protein where the factor Xa cleavage site is located at the N-terminal amino acid (serine 24) of mature STb, pMAL-IN plasmid was digested by *Xba*I and blunt ends were generated with mung bean nuclease. The recombinant plasmid was then

Fig. 1. Construction of plasmids pMAL-IN and pMAL-STb. Subcloning procedure for inserting *estB* into the pMAL-p vector. The *estB* gene was cloned into the *XbaI* and *HindIII* restriction sites of the pMAL-p polylinker. The pMAL-IN subclone was digested with *XbaI* and treated with mung bean nuclease. The DNA was then digested with *StuI* and ligated. Final subclones were screened with polyclonal anti-STb antibodies. *, Xa factor cleavage site.

digested with *StuI*, ligated, and electrotransformed into strain HB101 (Fig. 1). Clones expressing the MBP-STb fusion protein were screened immunologically with anti-STb polyclonal antibodies. A positive clone was selected and named pMAL-STb. The *malE-estB* construct was sequenced. The sequence determined for *estB* was present in the right orientation and in its integrity (Lee *et al.*, 1983). The *estB* gene coding for mature STb toxin was cloned downstream of the *malE* gene and its signal sequence. The pMAL-p vector carried a sequence coding for a factor Xa cleavage site, situated at the 5' end to the polylinker insertion sites, to create an MBP-STb fusion protein where the Xa cleavage site is located at the N-terminus of STb.

In this construct, the fused genes are controlled by the strong inducible *P_{tac}* promoter. After induction of *P_{tac}* by addition of IPTG, the final product of the pMAL-STb construct was detected immunologically as a MBP-STb

fusion protein of 47 kDa (Fig. 2a and b, lanes 6, 7). The MBP-STb protein was detected in total cell extract and in osmotic shock fluid, and reacted with anti-STb polyclonal antibodies (Fig. 2a, lanes 6, 7). In non-induced total cell extract, a faint band was observed at the same level as the MBP-STb fusion protein indicating a weak transcription of *malE-estB* even in the absence of induction (Fig. 2a, lane 5). The MBP-STb fusion protein was neither detected in strain HB101 nor in HB101 harbouring pMAL-IN (Fig. 2a, lanes 1, 2). The MBP-STb protein reacted with anti-MBP polyclonal antibodies, but the native MBP was barely detected in strain HB101 (Fig. 2b, lanes 1, 2).

The amount of MBP-STb protein produced by the pMAL-STb harbouring strain was evaluated after IPTG induction at various times. Increasing amounts of MBP-STb protein were observed up to 3 h after induction (Fig. 3, lanes 1 to 6). Longer induction of pMAL-STb did not

Fig. 2. Analysis of the MBP-STb fusion protein. (a) Immunoblot of total cell extracts using the anti-STb polyclonal antibodies of non-induced and induced cells. Lanes: 1, 2, total cell extracts of strain HB101, non-induced and induced, respectively; 3, 4, total cell extracts of pMAL-IN, non-induced and induced, respectively; 5, 6, total cell extracts of pMAL-STb, non-induced and induced, respectively; 7, osmotic shock fluid containing MBP-STb. Cells were grown at 37 °C in rich medium to $OD_{600} = 0.4$ and induced for 3 h with 0.3 mM-IPTG. (b) Immunoblot identical to that shown in (a) but using anti-MBP polyclonal antibodies. Molecular mass standards to the left of the gel.

increase the amount of MBP-STb produced. Three hours after IPTG induction, the MBP-STb fusion protein was the most abundant periplasmic component, constituting approximately 33% of total protein in the OSF as detected by gel scanning. On average, 1 litre of induced HB101 strain harbouring pMAL-STb yielded 3–4 mg MBP-STb protein in the OSF. STb comprises about 1/10 of the weight of the fusion protein and thus about 0.3–0.4 mg of STb toxin is present in 1 litre. MBP-STb fusion protein was purified from other components of the OSF by preparative electrophoresis using 12.5% (w/v) polyacrylamide gel and electroelution. OSF preparations containing MBP-STb protein were biologically active in the rat intestinal loop assay (Table 1). OSF was desalted on a Fast Desalting Column HR 10/10. When

Fig. 3. Kinetic study of induction of MBP-STb fusion protein. Immunoblot using anti-STb polyclonal antibodies. Lanes: 1 to 6, total cell extracts of pMAL-STb at 0, 30, 60, 90, 120 and 180 min after induction with IPTG, respectively; 7, 8 and 9, osmotic shock fluids 180 min after induction with IPTG from strain HB101, strain HB101 harbouring pMAL-p and strain HB101 harbouring pMAL-STb, respectively. Molecular mass standards to the left of the gel.

Table 1. *Biological activity in intestinal rat loop*

Figures are means $\pm$ SD of at least six determinations.

Strain	Sample	Ratio ($\pm$ SD)
HB101	Supernatant	0.04 $\pm$ 0.00
HB101 (pRAS1)	Supernatant	0.13 $\pm$ 0.01
HB101 (pMAL-p)	OSF	0.00 $\pm$ 0.00
HB101 (pMAL-STb)	OSF	0.125 $\pm$ 0.08

Fig. 4. MBP-STb fusion protein cleavage with Xa factor. Immunoblot using anti-STb polyclonal antibodies. Lanes: 1, osmotic shock fluid from pMAL-STb clone; 2, purified MBP-STb fusion protein; 3, MBP-STb fusion protein purified and cleaved with factor Xa. The arrow indicates STb polypeptide released by cleavage. Molecular mass standards to the left of the gel.

Fig. 5. Immunodot assay of desalted OSF. (a) Chromatogram of osmotic shock fluid desalted on a Fast Desalting Column HR 10/10. (b) Immunodot of fractions using anti-STb polyclonal antibodies. Numbers refer to collected fractions.

fractions were analysed by immunodot using anti-STb serum, peaks nos 1 and 3 were positive whereas peaks nos 2, 4 and 5 were negative (Fig. 5). The MBP-STb fusion protein, purified from the OSF by preparative electrophoresis and electroelution, was incubated with the Xa protease. Cleavage of MBP-STb yielded a peptide of approximately 5 kDa that reacted with anti-STb serum (Fig. 4, lane 3).

Discussion

Various gene fusion systems have been described for expression and purification of STb enterotoxin to provide a source of immunogen and/or biologically active toxin (Spandau & Lee, 1987; Handl *et al.*, 1988; Lawrence *et al.*, 1990; Urban *et al.*, 1990a, 1991). However, many of the fusion proteins engineered showed little biological activity and/or the STb toxin could not be recovered from the hybrid protein.

Here we describe the genetic construction of a *malE*-*estB* gene fusion for overexpression of STb toxin. The expression and biological properties of the fusion protein were analysed. A MBP-STb fusion protein with a factor

Xa cleavage site at the serine 24 position of the STb precursor protein was produced (Lee *et al.*, 1983; Picken *et al.*, 1983; Kupersztach *et al.*, 1990). Restriction mapping and sequence analysis confirmed the presence and proper orientation of the entire *estB* gene without its signal sequence.

One of the aims of this study was to overexpress the *estB* gene. Spandau & Lee (1987) showed that transcription of STb is regulated by a relatively weak constitutive promoter. Urban *et al.* (1990a) used a recombinant strain harbouring a cloned copy of the *estB* gene and replaced the endogenous STb promoter with several highly active promoters such as bacteriophage T7 promoter and P_{lac} . Construction of the hybrid plasmid named pMAL-STb, overexpressing STb enterotoxin, presents some advantages. The expression of MBP-STb is controlled by the strong, inducible P_{lac} promoter. IPTG induction resulted in production of a MBP-STb fusion protein of 47 kDa, detectable in total cell extract and in osmotic shock fluid. The synthesized fusion protein remained cell-bound and was found in *E. coli* periplasm. A kinetic study of induction with IPTG revealed that 3 h after induction, the fusion product represented approximately 33% of OSF protein. This amount was not increased by longer incubation. In the absence of IPTG induction, the *lacI* repressor gene kept expression of the fusion low (Fig. 2a lane 5). Recently, Aitken & Hirst (1992) engineered a set of fusion proteins containing maltose-binding protein and heat-stable enterotoxin STa (MBP-STa) to generate solid-phase antigens that can be used to monitor anti-STa antibody response. Similarly to our results, they showed the fusion protein to be the most abundant single component found in the periplasm. Urban *et al.* (1990b) showed by ELISA that natural STb-producing strains produce little STb in defined-medium cultures relative to production by a recombinant strain harbouring a cloned copy of the *estB* gene. Replacement of the endogenous STb promoter with several highly active promoters increased the yield of STb 10- to 20-fold over that obtained by an *E. coli* strain harbouring the recombinant *estB* gene, and correlated with intestinal secretory activity. Lawrence *et al.* (1990) cloned the STb gene in a high-expression vector pKC30 downstream from the strong pL promoter. They showed a 10- to 20-fold increase in messenger RNA of the strain containing the hybrid plasmid compared to a wild-type strain. However, there was little or no increase in enterotoxin activity as measured by the pig intestinal loop assay.

The MBP-STb protein can be easily purified from the OSF using preparative electrophoresis and electroelution. Moreover, the fusion protein can also be purified by affinity chromatography using amylose resin (data not shown). In pMAL-STb, the Xa site is at the

junction between the MBP gene with its signal sequence and the gene for mature STb toxin. This protease cleaves specifically after a four amino acid recognition sequence (Fig. 1). The mature STb 5 kDa toxin could be recovered after hydrolysis with factor Xa.

Obtaining biologically active toxin was of prime importance as one of the purposes of this construct is to obtain STb toxin for elucidation of its mechanism of action. Handl *et al.* (1988) fused the *estB* gene with the gene for protein A, but the purified protein A-STb fusion had very low biological activity when tested in the pig intestinal loop assay, and the STb toxin could not be recovered from the fusion protein. Urban *et al.* (1990a) also produced a fusion protein between STb and alkaline phosphatase. This fusion protein induced intestinal secretion in a rat intestinal ligated loop assay. In these studies, the STb toxin was recovered as a fusion protein. In addition, there was no way to obtain the native toxin.

To examine the toxic property of the pMAL-STb fusion protein, crude OSF preparations from strain HB101 harbouring pMAL-STb were tested in the rat intestinal loop assay (Table 1). The OSF preparation was active in the biological model. In our study, STb was identifiable either by bioassay or as a band of approximately 5 kDa either by silver staining or immunoblot after it was cleaved with factor Xa (Fig. 4). Thus, the STb moiety in the fusion protein was accessible to the anti-STb antibodies. In our study, OSF was biologically active, but intact MBP-STb molecules purified by gel filtration chromatography lacked enterotoxin activity (Fig. 5, peak 1). These results are in accordance with results published earlier by Handl *et al.* (1988) analysing the biological activity of protein A-STb in a pig intestinal loop assay. MBP-STb molecules are most probably spontaneously hydrolysed in OSF, and free STb molecules seem to be responsible for the biological activity of OSF in the rat intestinal loop assay. Thus, since MBP-STb has low biological activity as an intact molecule, the biological activity is likely to be associated with hydrolysed molecules. One possible explanation for the lack of activity of the fusion protein could be steric hindrance between the large carrier molecule and the low-molecular-mass toxin. More recently, a fusion protein between MalE and CD4, a specific receptor for HIV, (Clément *et al.*, 1989; Szmecman *et al.*, 1990) was shown to be transported within the periplasm as expected. However, the hybrid protein was partially degraded even in a protease-minus background.

Immunization of animals is likely to evoke antibody responses to both proteins of the fusion. The maltose-binding protein of *E. coli* may be useful as a carrier molecule for the poorly immunogenic STb toxin (Dubreuil *et al.*, 1991). The observation that the intact

fusion protein possessed markedly reduced or no toxicity compared to altered (hydrolysed) molecules suggests that it could be used to induce an STb-neutralizing response. This possibility should be studied in more detail.

Since MBP-STb constitutes approximately 33 % of the OSF total protein content for a yield of approximately 3–4 mg fusion protein per litre of induced culture, it is a promising way to obtain active STb in high quantity. However, this level of expression cannot be compared with that of the *E. coli* porcine strain used to purify STb (Dubreuil *et al.*, 1991), since the yield was not evaluated in the latter case. To this day, the STb toxin has remained difficult to obtain in pure form from culture supernatants, not only because of its small size and sensitivity to proteolysis, but also because of the small amount of toxin recovered from culture supernatants of *E. coli* strains. The MBP-STb protein may provide a source of mature and biologically active STb toxin for determination of the toxin's mode of action.

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