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Secretion of Methanol-Insoluble Heat-Stable Enterotoxin (ST_B): Energy- and *secA*-Dependent Conversion of Pre-ST_B to an Intermediate Indistinguishable from the Extracellular Toxin

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The methanol-insoluble, heat-stable enterotoxin of *Escherichia coli* synthesized by clinical strains or strains that harbor the cloned gene was shown to be an extracellular polypeptide. The toxin (ST_B) was first detected as an 8,100-M_r precursor (pre-ST_B) that was converted to a transiently cell-associated 5,200-M_r form. Proteolytic conversion of pre-ST_B to ST_B was shown to be inhibited by the proton motive force uncoupler carbonyl cyanide *m*-chlorophenylhydrazone and did not occur in a *secA* background. After ST_B was detected as a cell-associated molecule, an extracellular form with identical electrophoretic mobility became apparent. The results suggest that there is no proteolytic processing during the mobilization of ST_B from the periplasm to the culture supernatant. The determined amino acid sequence of ST_B coincides fully with the 48 carboxy-terminal amino acids inferred from the DNA sequence. The 23 amino-terminal residues inferred from the DNA sequence were absent in the mature toxin.

Enterotoxigenic *Escherichia coli* synthesizes the heat-labile and the heat-stable (ST) families of enterotoxins; these toxins have been shown to be responsible for secretory diarrhea in humans and animals (reviewed in references 1 and 30). STs have been classified as methanol soluble (ST_A) and methanol insoluble (ST_B) (3, 36), and these subdivisions correlate well with the inferred or known amino acid compositions of the toxins (18, 21, 27). The toxic activity of ST_A is resistant to proteases (6, 33), while ST_B is inactivated upon trypsin treatment (35). ST_As are 18- or 19-amino-acid extracellular enterotoxins that result from two independent proteolytic cleavages on a 72-amino-acid precursor (pre-pro-ST_A); the first cleavage yields a periplasmic 53-amino-acid pro-ST_A that is extracellularly processed to mature ST_A (29a). The three disulfide bridges formed by the six cysteine residues of ST_A are sine qua non for toxic activity (6, 10, 33). A structural model based on proton nuclear magnetic resonance has been proposed for this toxin (11, 24). It is also known that ST_A interacts with an enterocyte receptor that activates guanylate cyclase and results in increased intestinal secretion (8); in contrast to ST_A, very little is known about the mechanism of action and the export-secretion pathway of methanol-insoluble ST_B; its gene (*estB*) has been sequenced (21, 27), and the 71-codon open reading frame, when translated, is very different from the 72 residues of the precursor of ST_A (reviewed in reference 18). The first 23 amino-terminal amino acids inferred from the *estB* sequence have properties compatible with a signal peptide (21, 27), and it has been proposed that mature ST_B is a 48-residue molecule; it was unclear, however, whether ST_B is an extracellular polypeptide secreted into the medium like ST_A (16) or whether it is a periplasmic enterotoxin like heat-labile enterotoxin (26). In this communication, we show that mature ST_B is a 48-amino-acid extracellular polypeptide that corresponds to the previously inferred carboxy-terminal amino acid sequence; we also show that a single processing event on a precursor yields a transient periplasmic species that, without further apparent modifications, becomes extracellular ST_B.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *E. coli* strains used were HB101 (2), MC4100, and its temperature-sensitive *secA* mutant, MM52 (25). Also used were the porcine nontoxigenic strain *E. coli* 123 and strain 1790 (*estB*$^+$) (35). Plasmids pGP1-2, pT7-5, and pT7-3 have already been described (34) and were kindly provided by S. Tabor. A 375-base-pair *estB* DNA fragment lacking the natural promoter of the gene was excised from pCHL6 (21) by digestion with *Mnl*I-*Hin*dIII and then ligated to pT7-5 predigested with *Sma*I and *Hin*dIII. The resulting plasmid (pUD1) was isolated and cleaved with *Eco*RI-*Hin*dIII, and the *estB*-containing fragment was cloned into pT7-3 (predigested with the same enzymes) to yield pYK300. In this plasmid, *estB* precedes the β-lactamase gene and both are in the same orientation. In all radiolabeling experiments, we used low-sulfate C medium (17) supplemented with kanamycin (50 μg/ml) and ampicillin (50 μg/ml). To determine ST_B activity, the bacterial strains were grown on brain heart infusion broth (36).

Pulse-chase experiments and analysis of radioactive polypeptide. Strains HB101(pGP1-2)(pT7-3) and HB101(pGP1-2)(pYK300) were grown separately at 29°C in C broth; when the cultures reached a density of 5×10^8 cells per ml, they were shifted to 42°C for 15 min, 100 μg of rifampin per ml was added, and the incubation was continued for an additional 10 min. The cultures were then shifted to 37°C for 15 min. When the strains were MM52 and/or MC4100, the cultures were grown at 29 or 37°C before incubation at 42°C and then shifted back to 29 or 37°C. Radiolabeling of polypeptides was accomplished by incubation with [^{35}S] cysteine (15 μCi/ml). After the indicated pulse times, C

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TABLE 1. Extracellular localization of ST_B

Strain (phenotype)	% Total β-galactosidase activity[a]		% Total ST_B activity[a]	
	Cells	Supernatant	Cells	Supernatant
1790 (ST_B^+)	98	2	0.5	99.5
123 (ST_B^-)	99	1	ND	ND

[a] β-Galactosidase and ST_B activities were determined as described in Materials and Methods. ND, Not detectable.

medium supplemented with cold cysteine (8 mg/ml) was added and incubation was continued for the indicated chase times. The chase was stopped by bringing the suspension to 33 μg of chloramphenicol per ml, 133 mM sodium azide, and 6.6 mM 2,4-dinitrophenol (17, 31) (stop medium). Supernatants and cell pellets were obtained after centrifugation, and the pellets were suspended in ice-cold distilled water. When indicated, the cellular pellet was suspended in stop medium and sonicated (16); polypeptides from the supernatant and bacteria were precipitated with 10% cold trichloroacetic acid. Before trichloroacetic acid precipitation, unlabeled bacteria were added to the supernatant fraction to make the optical density at 540 nm equal to that of the cellular fraction. The polypeptides were suspended in sample buffer (17) and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10 to 15% acrylamide gradient gels (29a) but with an increase in the molarity of Tris to 0.75 M (9). When indicated, the gels were stained with Coomassie blue and/or the radioactive band was fluorographed after treatment with sodium salicylate as described previously (17).

Amino acid sequence determinations. A sample of purified ST_B peptide (S. Whipp et al., manuscript in preparation) was subjected to automated Edman degradation with an Applied Biosystems (Foster City, Calif.) 470A amino acid sequencer. Amino acids (phenylthiohydantoin derived) were identified by using an on-line 120A high-pressure liquid chromatography system. Both instruments used standard manufacturer-recommended programming and chemicals. No attempt was made to identify the cysteine residues, and they appeared as gaps in the sequence. No PTH amino acids were identified at the positions occupied by cysteine.

Biochemical and enterotoxin assays. β-Galactosidase was assayed with *o*-nitrophenyl-β-D-galactopyranoside (16), and ST_B activity was determined in intestinal loops of pigs as described previously (36).

RESULTS

Extracellular localization of ST_B. Enterotoxigenic *E. coli* synthesizes periplasmic (heat-labile) and extracellular (ST_A) enterotoxins. To determine the localization of ST_B, a natural ST_B^+ ST_A^- LT^- enterotoxigenic *E. coli* strain, 1790, and an ST_B^- control strain, 123, were grown in the conditions used to assay ST_B. After centrifugation, supernatant and cell pellet were tested for ST_B activity and intracellular β-galactosidase. In both ST_B^+ and ST_B^- strains, β-galactosidase was associated with the cellular fraction while ST_B was found preferentially in the supernatant of the ST_B^+ strain (Table 1). Thus, ST_B is an extracellular polypeptide in its natural *E. coli* host.

Transient cellular *estB* gene product with the same apparent molecular weight as an extracellular product. As described in Materials and Methods, the gene that encodes ST_B (*estB*) was cloned into plasmid pT7-3 to yield pYK300. Expression

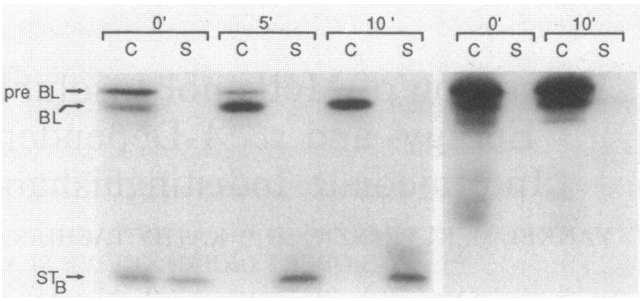

FIG. 1. An intracellular form of ST_B shows the same electrophoretical mobilities as the extracellular toxin. Cultures of ST_B-producing strain HB101(pGP1-2)(pYK300) and isogenic ST^- strain HB101(pGP1-2)(pT7-3) were pulsed for 2 min with [^{35}S]cysteine and chased for the times indicated. After centrifugation, cell pellets (C) and supernatants (S) were separately submitted to SDS-PAGE and the gel was fluorographed. Left panel, ST_B^+; right panel, ST_B^-. Indicated are the intracellular precursor (pre BL) and periplasmic mature (BL) forms of β-lactamase. In the left panel, the ST_B band in the cellular fraction obtained immediately after the pulse (0′ chase) was not visible after a 5-min chase and all of the detectable ST_B appeared in the supernatants. These bands were absent in the isogenic *estB* samples (right panel).

of this system under the conditions described did not affect the localization of ST_B and β-galactosidase. Upon induction of the T7 RNA polymerase system and [^{35}S]cysteine pulse-labeling of the control [HB101(pGP1-2)(pT7-3)] and ST_B^+ [HB101(pGP1-2)(pYK300)] strains, the precursor and mature forms of β-lactamase were clearly visible (Fig. 1); in the pulse of the *estB* strain, a 5,500-M_r band was detected within the bacteria (Fig. 1, left panel). After chasing, the β-lactamase precursor-product relationship decreased while the 5,500-M_r band present in *estB* bacteria was no longer detected; instead, an extracellular band with the same electrophoretic mobility was observed. When the pulsed cells were mixed with chased supernatant, the 5,500-M_r species appeared as a single band (data not shown). These bands were absent from pulse-chase samples obtained from the supernatant and/or the cellular pellet of the ST_B^- strain (Fig. 1, right panel). Thus, a cellular precursor of ST_B had the same apparent molecular weight as an extracellular ST_B species.

This cell-associated precursor form of ST_B was found in soluble cellular fractions and not in the membrane pellet; this was concluded on the basis of analysis of SDS-PAGE of supernatants and pellets that resulted from centrifugation (171,000 × *g*, 1 h, 4°C) of sonic cell extracts of radiolabeled *estB* bacteria. Mature ST_B was found together with periplasmic β-lactamase and its cytoplasmic precursor in the soluble fraction (Fig. 2). The precursor of β-lactamase was detected in the pellet, and in this fraction, ST_B was absent. The samples in these experiments were reduced with dithiothreitol, and a fraction of each was treated with 176 mM iodoacetamide. The carboxy-amidated samples yielded sharper bands, probably by avoiding disulfide formation during manipulation and electrophoresis of the samples.

Accumulation of pre-ST_B by treatment with CCCP. The proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) dissipates the membrane potential of *E. coli* (5) and has been shown to cause accumulation of precursors of periplasmic and outer membrane proteins. To determine whether ST_B shares the general export pathway blocked by CCCP and to determine whether a cellular precursor other than the one shown in the preceding section exists, CCCP inhibition experiments were performed. CCCP caused a

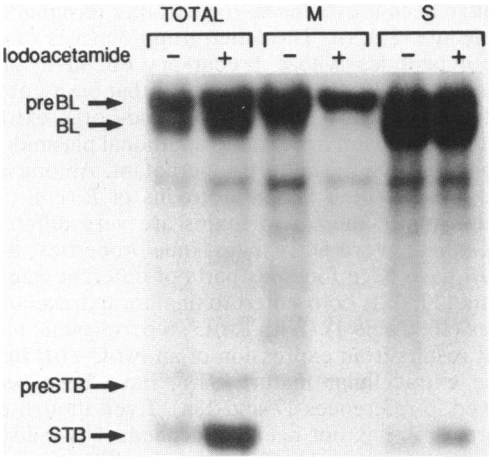

FIG. 2. The cell-associated form of ST_B is in a soluble fraction. The bacteria of an *estB* culture labeled for 2 min with [^{35}S]cysteine were sonically disrupted and centrifuged (150,000 × *g*, 1 h, 4°C); the pellet (M, membranes) and the soluble fraction (S) were incubated with 80 mM dithiothreitol, and half of each sample was treated with 157 mM iodoacetamide. Unfractionated (TOTAL) membrane (M), and soluble (S) fractions were submitted to SDS-PAGE and fluorography. ST_B was apparent in the unfractionated and soluble samples, while pre-ST_B was detected in the unfractionated sample and in the membrane fraction.

reduction of the rate of protein synthesis and prolonged the life of the precursor of β-lactamase (Fig. 3). Treatment with CCCP allowed detection of a newly observed ST_B species that was larger (with an apparent M_r of 8,100) than the cellular precursor previously identified (M_r, 5,500). The 5,500-M_r form of ST_B was not detected in CCCP-treated cultures (Fig. 3B). Without CCCP, the two cellular ST_B-related species were detected immediately after the pulse, and upon chasing, they became less prominent in the cellular fractions. As the chase continued, the band intensity of the extracellular polypeptide increased (Fig. 3A). The larger species was associated with the membrane fraction of sonicated bacteria (Fig. 2).

These results are taken to indicate that a cellular precursor (pre-ST_B; M_r, 8,100) larger than ST_B (M_r, 5,500) was apparent only after a short pulse and its cellular residency was increased by CCCP treatment. Treatment with CCCP did not increase the half-life of the 5,500-M_r cellular ST_B precursor. Experiments designed to determine the energy-dependent conversion of cellular to extracellular ST_B showed that this event took place in the presence of CCCP or 2,4-dinitrophenol (data not shown). Therefore, conversion of cellular ST_B to extracellular toxin did not depend on membrane potential or oxidative phosphorylation.

***secA*-dependent conversion of pre-ST_B to cellular ST_B.** Most periplasmic and outer membrane proteins require the *secA* gene product for conversion of the precursor to the mature gene product. To establish whether conversion of pre-ST_B to cellular ST_B depends on *secA*, the ST_B-T7 expression system was transformed into MC4100 and its temperature-sensitive *secA* mutant, MM52. Analysis of the expression of these systems showed that at 29°C, the permissive temperature, processing of pre-β-lactamase and pre-ST_B in strain MM52 was indistinguishable from that of the wild-type *secA*$^+$ strains [HB101(pGP1-2)(pYK300) (Fig. 3) and MC4100(pGP1-2)(pYK300) (the parental strain of MM52; data not shown)]. At the nonpermissive temperature (37°C), neither pre-β-lactamase nor pre-ST_B was processed in the *secA* background of strain MM52 (Fig. 4). Thus, at this temperature, extracellular ST_B was not observed. These results indicate that conversion of pre-ST_B to cellular ST_B is dependent on the *secA* gene product.

Amino acid sequence of mature ST_B. Analysis of purified ST_B by SDS-PAGE revealed a single silver- or Coomassie blue R250-stained band. A sample of this ST_B solution was subjected to automated Edman degradation. The first 23 amino acids inferred from the gene sequence were absent in the ST_B sequence; thereafter, each residue identified (Fig. 5) was in accord with the predicted amino acid sequence; the yield of glutamine in cycle 3 was 285 pmol, and the measured

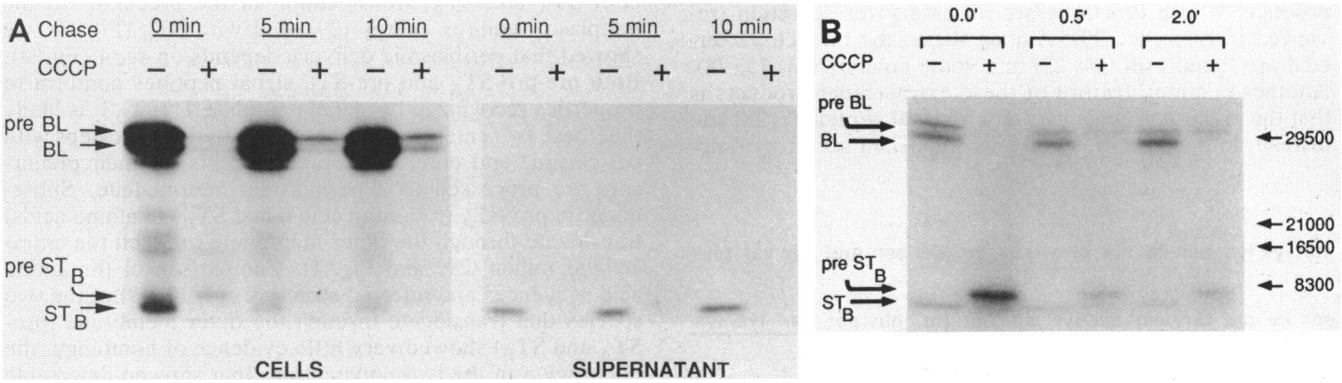

FIG. 3. Inhibition of ST_B processing and secretion by CCCP. ST_B^+ cultures [HB101(pGP1-2)(pYK300)] preincubated for 5 min at 37°C with (+) or without (−) 40 μM CCCP were pulsed for 5 min with [^{35}S]cysteine and chased for the indicated times; cells and supernatants were trichloroacetic acid precipitated together (B) or after they had been separated by centrifugation (A), and then they were submitted to SDS-PAGE and fluorography. The precursors of β-lactamase (pre BL) and ST_B (pre ST_B) were the prevailing species detected in the cellular samples treated with CCCP. In the untreated cellular samples, pre-β-lactamase (pre-BL) was converted with chase time to β-lactamase (BL), and pre-ST_B and ST_B were chased out of the bacteria. Without CCCP, ST_B was the only species identified in the supernatant. No extracellular ST_B was detected in the CCCP-treated samples. In the unfractionated culture, it was apparent that CCCP caused a reduction in the rate of conversion of pre-ST_B to ST_B; in this gel (B), equal counts were applied in each line. In the gel in which cells were separated from the supernatant (A), equivalent volumes were applied. The molecular weight markers were, in decreasing order, carbonic anhydrase, β-lactoglobulin, lysozyme, and bovine trypsin inhibitor. The mobilities of the prestained molecular weight markers and their relative molecular sizes were determined in relation to those of the unstained species, visualized with Coomassie blue R250.

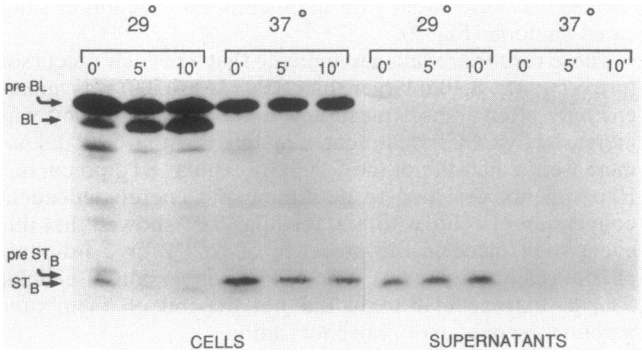

FIG. 4. Dependence on the *secA* gene product for processing and secretion of pre-ST_B. A temperature-sensitive *secA* mutant (MM52) harboring *estB* (pGP1-2 and pYK300) was preincubated for 2 generations at the permissive (29°C) or nonpermissive (37°C) temperature. When the cultures reached 5×10^8 cells per ml, they were radiolabeled for 2 min with [^{35}S]cysteine and chased for the indicated times. After centrifugation, the cells and supernatants were trichloroacetic acid precipitated, suspended in sample buffer, and fractionated by SDS-PAGE. At the nonpermissive temperature, only the precursors of β-lactamase (pre BL) and ST_B (pre ST_B) were detected and ST_B was absent from the supernatant. In contrast, at the permissive temperature, processing of pro-β-lactamase and pre-ST_B was apparent and extracellular ST_B was visible.

repetitive yield was 90.4%. No attempt was made to identify the cysteine residues, and no PTH amino acids were identified in positions 10, 21, and 36; in these positions, as well as in position 48, the DNA sequence predicts cysteine residues. This indicates that residues 1 to 23 inferred from the DNA sequence are not present in mature extracellular ST_B.

DISCUSSION

Very few extracellular peptides are naturally secreted by *E. coli*. Of these, most are plasmid encoded. The plasmid-encoded extracellular polypeptides fall into two discrete groups. (i) In one group, the plasmid that encodes the extracellular structural gene product also encodes other gene products whose functions are necessary for secretion (reviewed in reference 29). Among these, the best characterized are hemolysin (14, 22) and some colicins (13, 15, 28). Another common feature of these extracellular products is that the precursor molecules lack a signal peptide (7, 28) and instead, in the case of hemolysin, important secretory functions have been attributed to the carboxy-terminal region of the molecule (23). (ii) The other group consists of extracellular polypeptides whose precursors do have an amino-terminal extension, the signal peptide, that is absent from the mature form. In this group, the plasmid-borne extracellular gene products do not necessitate additional plasmid-encoded functions to reach the culture supernatant. Among members of this group are the ST enterotoxins of *E. coli* (29a; this communication); these two toxins are very different polypeptides that nevertheless share some properties. Both *estA* and *estB* have been found as parts of different transposable elements (20, 32); both enterotoxins are extracellular polypeptides (16; Table 1). With *estA3*, a periplasmic intermediate that results from expression of an *estA3-eltB* fusion (17) and the extracellular mature ST_{A3} have been sequenced (reviewed in references 17 and 18a). Even though extracellular pro-ST_{A3} has not been sequenced, it is indistinguishable from periplasmic pro-ST_A as judged by its electrophoretic mobility and its retention time in reverse-phase high-pressure liquid chromatography (Y. Kupersztoch and K. Tachias, unpublished data). It is therefore reasonable to assume that periplasmic and extracellular pro-ST_A have the same primary structure. With *estB*, mature extracellular ST_B was sequenced (Fig. 5); a cell-associated form with identical electrophoretic mobility has not been sequenced. This cell-associated intermediate appeared always as a soluble peptide and was not membrane bound (Fig. 2); very likely, it is a periplasmic species. Therefore, we propose that like secretion of ST_A, secretion of ST_B is via a periplasmic intermediate that has the same primary structure as mature extracellular ST_B. These periplasmic export intermediates result from cleavage of 19- and 23-amino-acid signal peptides of 72-amino-acid pre-pro-ST_A (17, 29a) and 71-residue pre-ST_B (Fig. 5) precursor molecules, respectively. When *bla*$^+$ *estB*$^+$ cultures were pulse-chased in the presence of the proton motive force uncoupler CCCP, there was a reduction in the rate of protein synthesis and in the rate of conversion of pre-β-lactamase and pre-ST_B to β-lactamase and ST_B, respectively (Fig. 3); this effect was seen clearly in the unfractionated culture (Fig. 3B). Furthermore, no processed ST_B was detected cellularly or extracellularly (Fig. 3A). In both ST_A and ST_B, translocation of the precursor to the periplasm requires energy (29), and with ST_B (Fig. 3), we showed that periplasmic delivery depends on *secA* (Fig. 4). Both pre-pro-ST_A and pre-ST_B signal peptides conform to properties recognized by signal peptidase 1; thus, it is likely that these two enterotoxins share the initial export steps with periplasmic and outer membrane proteins, as their precursors are processed to a periplasmic intermediate. Subsequently, pro-ST_A (53 amino acids) and ST_B (48 amino acids) translocate through the outer membrane to reach the extracellular milieu (29a and Fig. 1). Comparison of the amino acid sequences and inferred structures (4, 12, 19) of the two species that translocate through the outer membrane (pro-ST_A and ST_B) showed very little evidence of homology; the only region of the two polypeptides that showed detectable homology were pro-ST_A residues 41 to 50 (IAKkSnKsGp) and ST_B residues 39 to 48 (IAKeScKkGf) (shown in capitals are amino acids common to both polypeptides). It is conceivable that this motif is important as a targeting domain in the translocation of these two polypeptides through the outer membrane; additionally, the secreted forms of both toxins are basic peptides with high cysteine and serine contents. We have attempted, by site-directed mutagenesis of *estA3*, to define regions that are necessary for secretion. The only cases in which an ST_A-derived peptide remained preferen-

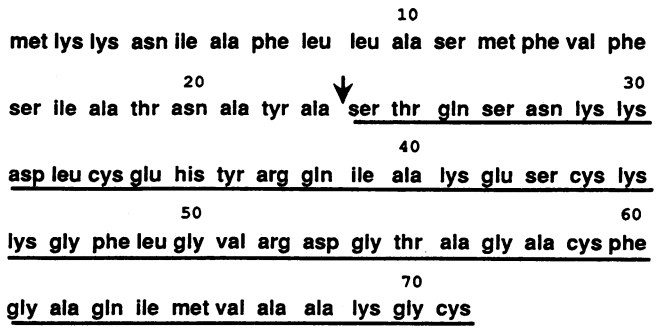

FIG. 5. Inferred (from the DNA sequence [20, 27]) and determined (underlined) amino acid sequences of ST_B polypeptides (GenBank accession no., A33063). The arrow indicates the cleavage site for conversion of pre-ST_B to ST_B.

tially cell associated have been correlated with insertion of additional amino acids at the carboxy terminus of the toxin (17, 18). (This region is also similar in *estB*: *estA* [AvtGCy], *estB* [AakGC].) Thus, it is likely that in pro-ST_A the natural carboxy terminus has to be terminal.

While the mature form of ST_B has electrophoretic mobility undistinguishable from that of the periplasmic form of the toxin, pro-ST_{A3} undergoes extracellular maturation that results in 34-residues (pro) and the active 19-amino-acid toxin (ST_A) (29a). ST_A and ST_B are apparently secreted by similar mechanisms, but ST_A is proteolytically matured extracellularly in an event independent of its secretion.

To confirm the identity of the secreted form of ST_B, we determined the amino acid sequence of the mature extracellular toxin. The sequence and site of processing were correctly predicted on the basis of the DNA sequence (21, 27). However, the mechanism by which the molecule exits the bacteria and the events that render it enterotoxic remain unclear. Experiments that address both issues are in progress.

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