

Two precursors of the heat-stable enterotoxin of *Escherichia coli*: evidence of extracellular processing

J. K. Rasheed,[†] L.-M. Guzmán-Verduzco and

Y. M. Kupersztoch*

Department of Microbiology, The University of Texas,
Southwestern Medical Center, 5323 Harry Hines
Boulevard, Dallas, Texas 75235, USA.

Summary

Expression of the gene of the methanol-soluble, heat-stable enterotoxin of *Escherichia coli* (ST_A) allowed the identification by SDS-PAGE of a cell-associated 7500 Dalton ST_A -related peptide; when similar experiments were performed with a phosphate buffer SDS-PAGE system, an additional M_r 9800 band became apparent. The 9800 Dalton form, pre-pro- ST_A , accumulated as an intracellular species when the experiments were performed in the presence of the proton ionophore CCCP (carbonylcyanide *m*-chlorophenylhydrazone); by pulse-chase experiments, it was shown that pre-pro- ST_A became a periplasmic M_r 7500 pro- ST_A and this form was chased to the culture supernatant; periplasmic and extracellular pro- ST_A showed the same electrophoretic mobility. A short time after the pulse, pro- ST_A was converted extracellularly to mature ST_A (M_r 4500). It is proposed that ST_A is synthesized as pre-pro- ST_A , a 72-amino-acid peptide that is subsequently cleaved between amino acids 19 and 20 as it is translocated across the inner membrane. The resulting 53-amino-acid pro- ST_A is first detected in the periplasm and is then secreted to the culture supernatant. Pro- ST_A is cleaved extracellularly to yield mature ST_A (M_r 4500).

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) elaborate at least two types of enterotoxins which induce the clinical

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; TCA, trichloroacetic acid; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; pro- β L, pro- β -lactamase; β L, β -lactamase; ST_A , methanol-soluble heat-stable enterotoxin; *estA*, ST_A gene and *estA3*, allele 3 of *estA*; LT, heat-labile enterotoxin; *eltA* and *eltB*, A and B genes of LT. For simplicity, pre-pro- ST_A , pro- ST_A , and ST_A are referred to as the precursors and mature forms of ST_A .

Received 14 August, 1989. *Present address: Hospital Infections Program, Antimicrobics Investigations Branch, Centers for Disease Control, Atlanta, Georgia 30333, USA. *For correspondence. Tel. (214) 688 2210.

symptoms of secretory diarrhoeal disease in both humans and domestic animals: (i) the high molecular weight, cholera toxin-like, heat-labile (LT) and (ii) the low molecular weight, heat-stable (ST) families of enterotoxins (Sack, 1975; Betley *et al.*, 1986). STs have been classified as methanol-soluble (ST_A) and methanol-insoluble (ST_B) (Burgess *et al.*, 1978; Whipp *et al.*, 1981); these classifications also correlate with the inferred or known amino acid composition and with the biological properties of these toxins. ST_A s are peptides of 18–19 amino acids and are biologically active in both the suckling mouse and the pig ligated ileal loop models. The three ST_A alleles (*estAs*) sequenced to date (So and McCarthy, 1980; Moseley *et al.*, 1983; Sekizaki *et al.*, 1985; Stieglitz *et al.*, 1988; Guzmán-Verduzco and Kupersztoch, 1989) show a highly conserved 72-amino-acid open reading frame and, in all cases studied, the active toxins are 18- or 19-amino-acid extracellular polypeptides with six cysteine residues (Aimoto *et al.*, 1982; Lazure *et al.*, 1983; Ronnberg *et al.*, 1983; Takao *et al.*, 1983; Saeed *et al.*, 1984; Thompson and Gianella, 1985). ST_A s are not naturally immunogenic, do not react with conventional protein stains, do not precipitate with trichloroacetic acid (TCA) or with perchloric acid, and, under certain conditions, the toxins display unexpected electrophoretic mobilities (Rasheed *et al.*, 1988). These properties have hindered the characterization of the metabolic steps which must occur as the toxin is mobilized through the bacterial compartments as it exits the cell.

The first 19 amino acids of the 72-amino-acid peptide (pre-pro- ST_A), inferred from the nucleotide sequence of *estA3*, comprise a conventional leader peptide (von Heijne, 1985). We hypothesized (Guzmán-Verduzco *et al.*, 1983; Stieglitz *et al.*, 1988) that the initial 19 residues of the 72 amino acids that constitute pre-pro- ST_A would be cleaved by a signal peptidase (Wickner and Lodish, 1985), allowing the resulting 53-amino-acid protoxin (pro- ST_A) to translocate through the inner membrane to the periplasm. The localization of an ST_A -intermediate in the periplasm and the elimination of a 19-amino-acid signal sequence are supported by the discovery of a thermo-activatable form of ST_A in this compartment (Guzmán-Verduzco *et al.*, 1983) and by the studies on the processing of an ST_A -LT_B hybrid (Guzmán-Verduzco and Kupersztoch, 1990, accompanying paper); in the ST_A -LT_B fusion experiments a periplasmic polypeptide was sequenced and shown to

lack the first 19 amino-terminal amino acids. The final maturation of ST_A was thought to take place in the periplasm via a second proteolysis that yields pro (34 residues) and ST_A (19 residues).

In this report, we describe the preferential *in vivo* radiolabelling of the *estA3* gene product and its export intermediates. We show the presence of an intracellular precursor of ST_{A3} (pre-pro-ST_A) that is chased to a smaller intermediate (pro-ST_A) which is first detected in the periplasm; this same species exits the cell and extracellularly undergoes a proteolytic cleavage that results in the mature enterotoxin.

Results

Detection of a periplasmic precursor of ST_A (pro-ST_A)

The open reading frame of *estA3* predicts a 72-amino-acid polypeptide, whereas the extracellular mature ST_{A3} is a 19-amino-acid, cysteine-rich peptide. When cultures of strains RR1 *estA3*⁺ (Fig. 1B) and *estA3*⁻ (Fig. 1A) were pulse-labelled with [³⁵S]-cysteine, chased and the whole-cell lysed and analysed by fluorography of SDS-PAGE

fractionated polypeptides, both strains showed the efficient chase of the β -lactamase precursor (M_r 29 000) to the mature periplasmic form of the enzyme (M_r 27 000). In the fluorogram of the ST_A-producing strain (Fig. 1B), an additional M_r 7500 band was seen; this band was shown to be transiently cellular, since after a 5-min chase, it is no longer apparent in whole-cell lysates. In the same electrophoretic system, mature ST_{A3} was previously characterized as a 4500 Dalton polypeptide (Rasheed *et al.*, 1988). The larger size of the *estA3*-encoded polypeptide (M_r 7500) and its transient cellular association suggested that it was a precursor of ST_A. It was of interest to define the cellular compartment in which this transient form of ST was localized.

A periplasmic location of this transient form of ST_{A3} was shown by experiments in which cells were first converted into spheroplasts and then labelled. The supernatant of the spheroplast suspension could be considered analogous to the periplasm of intact cells. The precursor of β -lactamase was found within the spheroplasts of both the control (Fig. 1C) and ST_A-producing (Fig. 1D) strains, whereas the mature form of β -lactamase was found in the supernatants of both strains (Fig. 1C and 1D). However,

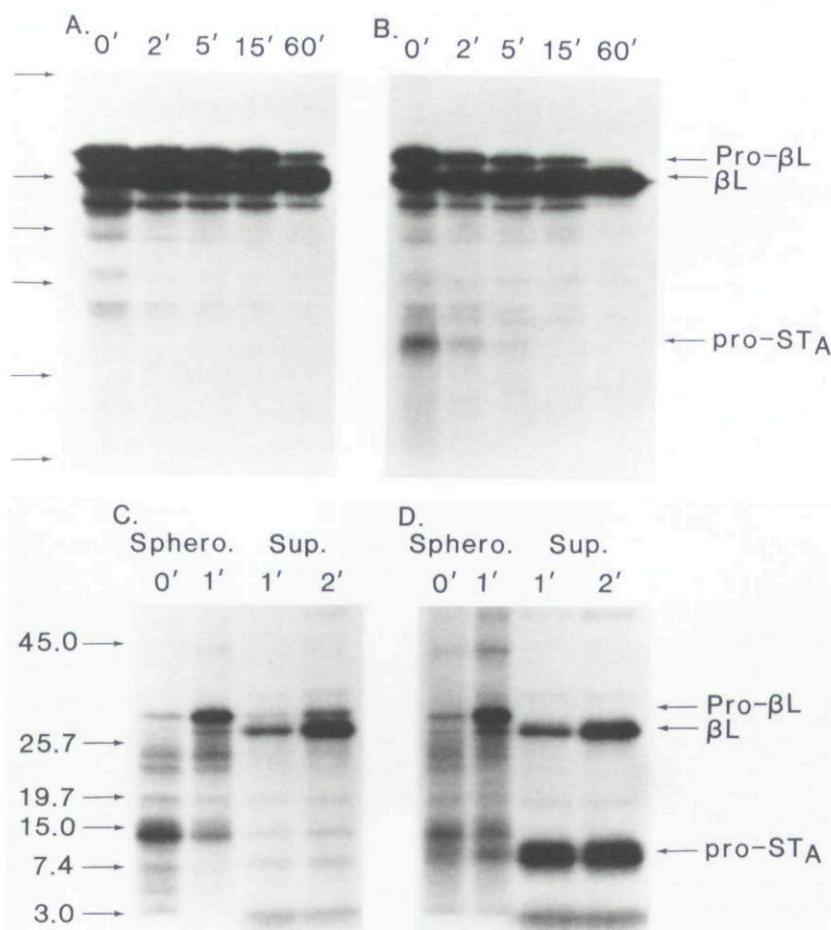


Fig. 1. A and B. Detection of [³⁵S]-cysteine-labelled ST_A precursor in whole-cell lysates. ST_A⁻ control strain (A) and ST_A-producing strain (B) were grown, induced, [³⁵S]-cysteine pulsed for 1 min and chased for the times shown. The harvested cells were analysed by SDS-PAGE and fluorography. Mature β -lactamase (β L) (M_r 27 000) and its precursor (pro- β L) (M_r 29 000) are indicated. This precursor of ST_A (pro-ST_A) is seen only within the ST_A-producing cells (B) out of which it is chased with time. Pre-stained molecular weight standards are, in decreasing order, ovalbumin, α -chymotrypsinogen, β -lactoglobulin, lysozyme, bovine trypsin inhibitor, and insulin.

C and D. Localization of ST_A-precursor in the supernatant of a labelled spheroplast suspension. The ST_A⁻ control strain (C) and ST_A-producing strain (D) were induced, converted into spheroplasts, [³⁵S]-cysteine-labelled for 1 min, and chased for the indicated times; the spheroplasts (Sphero.) and supernatant (Sup.), obtained after centrifugation were TCA precipitated and analysed by SDS-PAGE. The intracellular precursor of β -lactamase (pro- β L) and the mature periplasmic β -lactamase (β L) are indicated. The precursor of ST_A (pro-ST_A) is present only in the supernatant of the ST_A-producing strain (D). After chasing, supernatants of control (C) and the ST_A-producing (D) strains show the accumulation of mature β -lactamase (β L). Molecular weight standards are the same as above.

the supernatant of the spheroplasts of the ST_A-producing strain showed an additional M_r 7500 band (Fig. 1D) that was not detected in a similar sample of the control strain (Fig. 1C). To confirm that this precursor form of ST_A (pro-ST_A) was indeed localized in the periplasm, induced whole cells were labelled with [³⁵S]-cysteine and then fractionated into spheroplast and periplasm (Fig. 2A). It is clearly seen in Fig. 2A that pro-ST_A is mainly found as a transient periplasmic species. Mature β -lactamase can be seen to accumulate within the periplasm while pro-ST_A is being chased out of this compartment. In contrast, when spheroplasts were first formed and then pulsed and chased for over a period of 6 min, the pro-ST_A band found in estA3⁺ supernatants (Fig. 2C) did not decrease in intensity. These results indicate that in the fractionated periplasm, pro-ST_A is not processed whereas in whole cells, pro-ST_A exists transiently as a periplasmic form of ST. The disappearance of pro-ST_A from the periplasm could be due to its translocation to a different compartment, or its conversion to a smaller periplasmic form that is

not detected by the methods employed. The former alternative was found to be correct, as demonstrated experimentally in a following section (*Extracellular processing of pro-ST_A to mature ST_A*).

Detection of an intracellular precursor of ST_A (pre-pro-ST_A)

We had previously proposed (Guzmán-Verduzco *et al.*, 1983) that ST_A becomes extracellular through two independent processing steps with the formation of intracellular and periplasmic export intermediates. The detection of only one cellular form of ST_A could suggest that a single precursor and a single processing step were necessary and sufficient for the extracellular delivery of ST_A. Alternatively, it was also possible that under the experimental conditions used, the cleavage of the signal peptide could occur so rapidly that the 72-amino-acid precursor might not be detected (Ito, 1982).

Therefore, to slow down the processing by signal peptidase, pulse-chase experiments were performed in the presence of CCCP, a proton ionophore which dissipates the membrane potential of *E. coli* (Daniels *et al.*, 1981) causing the accumulation of precursors containing signal peptides. The initial experiments with CCCP showed a broader band in the region equivalent to pro-ST_A (M_r 7500), but did not distinguish additional ST_A-related peptides (not shown). It was then considered that the electrophoretic conditions employed did not allow the resolution of the two hypothetical ST_A precursors. Therefore the electrophoretic conditions were changed, to conditions shown to resolve related peptides with small differences in their molecular weight (Hayashi and Wu, 1985). The samples were reduced, carboxyamidated, and the polypeptides separated by SDS-PAGE using a 0.1 M NaH₂PO₄ buffer (Inouye and Guthrie, 1969). As shown in Fig. 3A, lane 3, an M_r 9800 band was detected in whole-cell lysates. This species migrates slower than the

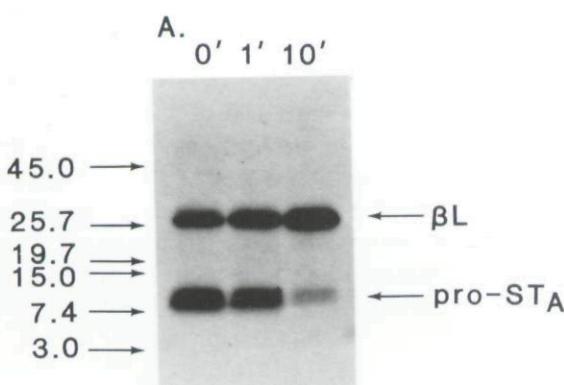


Fig. 2. A. Localization of pro-ST_A within the periplasm of labelled whole cells. After induction, the culture was pulsed with [³⁵S]-cysteine for 1 min and samples taken at the indicated chase times, the periplasm isolated, TCA precipitated and analysed by SDS-PAGE and fluorography.

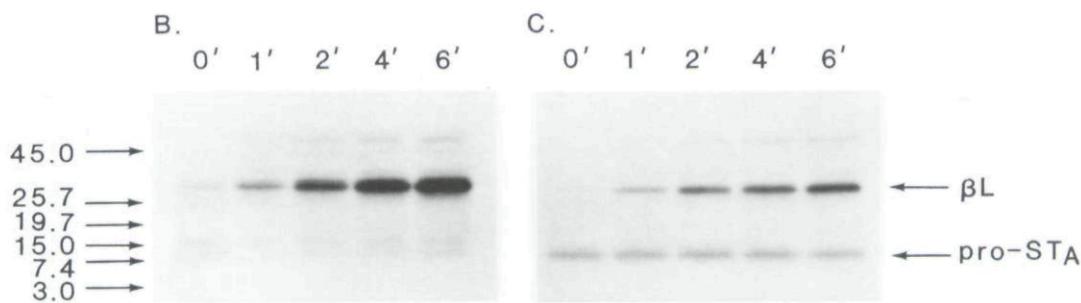
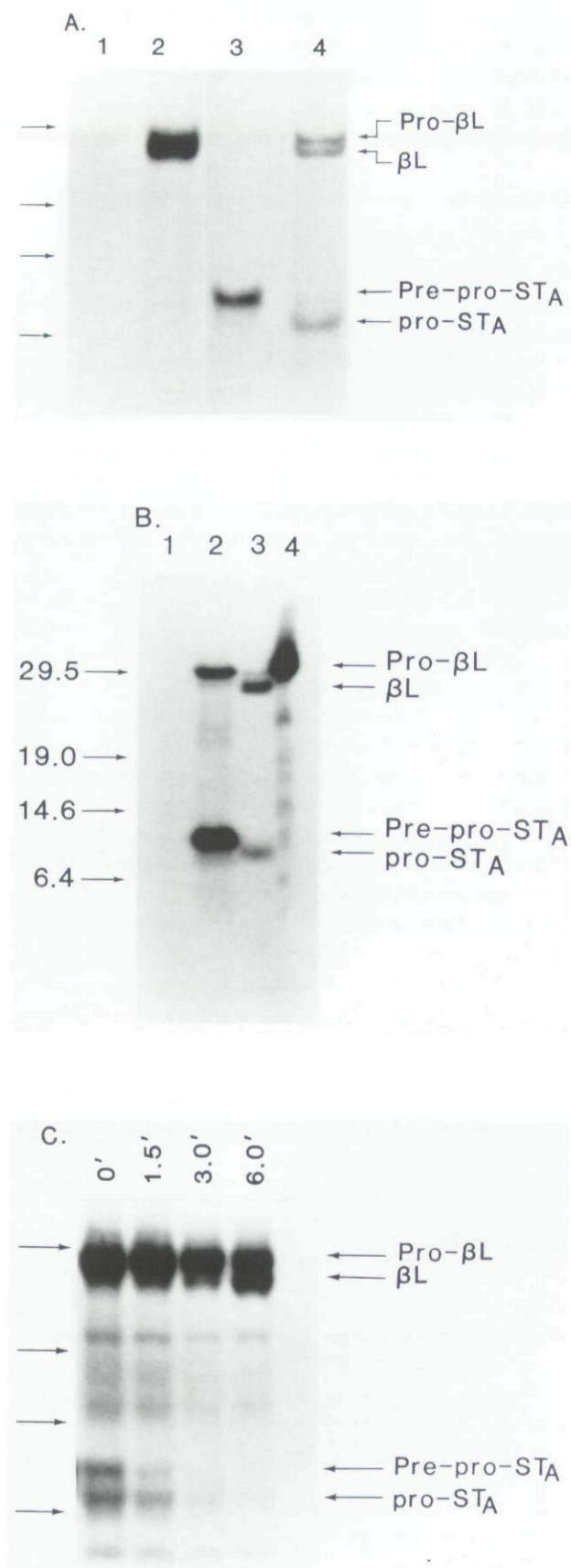


Fig. 2. B and C. Lack of processing of pro-ST_A in the supernatant of labelled spheroplasts. The ST_A⁻ control strain (B) and ST_A⁺ strain (C) were converted into spheroplasts which were pulsed for 1 min with [³⁵S]-cysteine and chased for the times shown. Supernatants and spheroplasts were separated, the supernatants TCA-precipitated and analysed by SDS-PAGE and fluorography. The mature forms of β -lactamase (βL) and pro-ST_A are indicated. Molecular weight markers are as described in Fig. 1.



band of pro-ST_A seen in the absence of CCCP (Fig. 3A, lane 4) and neither band is present in the similarly treated estA3⁻ control strain (Fig. 3A, lanes 1-2). These results show the presence of two cellular precursors of ST_A and strongly suggest that the M_r 9800 form corresponds to pre-pro-ST_A where pre is a signal peptide. To determine the localization of the 9800 Dalton species precursor and to confirm the periplasmic localization of the M_r 7500 form, cells were exposed to CCCP, labelled and fractionated into spheroplast and periplasm; pre-pro-ST_A and pro-β-lactamase appeared predominantly within the spheroplasts of the ST_A-producing strain (Fig. 3B, lane 2). In the absence of CCCP during labelling, mature β-lactamase and pro-ST_A were detected in the periplasm of the estA3⁺ strain (Fig. 3B, lane 3). When a similar culture was exposed to 50 μM CCCP after labelling, CCCP itself did not affect either the electrophoretic migration or the localization of these peptides. These results support the periplasmic and intracellular localization of pro-ST_A and pre-pro-ST_A, respectively, and indicate that the conversion of pre-pro-ST_A to pro-ST_A is energy-dependent.

The transient nature of the two ST_A precursors was also demonstrated by pulse-chase experiments similar to the one shown in Fig. 1B, in which phosphate buffer replaced Tris buffer, and the samples were carboxyamidated after their reduction. The results of one such experiment are shown in Fig. 3C. Within the bacteria, immediately after the pulse, the two species, pre-pro-ST_A and pro-ST_A, are clearly seen. During the chase, first pre-pro-ST_A becomes undetectable and subsequently pro-ST_A disappears from the bacteria. These data suggest that there is a precursor-product relationship between pre-pro-ST_A and pro-ST_A.

Fig. 3. A and B. Cellular localization and energy-dependent conversion of pre-pro-ST_A and pro-ST_A. After induction, the culture was split into two aliquots, 50 μM CCCP was added to one, and both fractions were incubated at 29°C for 15 min. CCCP-treated cells were labelled with [³⁵S]-cysteine for 5 min. Untreated cells were pulse-labelled for 1 min and chased for 1 min. The untreated samples were supplemented with 50 μM CCCP before electrophoresis. Whole cells (A) or spheroplast and periplasm (B) were analysed by SDS-PAGE in sodium phosphate buffer, and fluorographed as described in the *Experimental procedures*.
A. Whole cells. Lane 1, ST_A⁻, CCCP-treated; lane 2, ST_A⁻, untreated; lane 3, ST_A⁺, CCCP-treated; lane 4, ST_A⁺, untreated.
B. Cellular fractionation. Lane 1, ST_A⁺, CCCP-treated, periplasm; lane 2, ST_A⁺, CCCP-treated, spheroplasts; lane 3, ST_A⁺, untreated, periplasm; lane 4, ST_A⁺, untreated, spheroplasts. The precursor of β-lactamase (pro-βL), mature β-lactamase (βL), pre-pro-ST_A and pro-ST_A are indicated. Molecular weight markers, visualized by Coomassie brilliant blue staining are, in decreasing order, carbonic anhydrase, α-lactoglobulin, lysozyme, and bovine trypsin inhibitor.
C. Two cellular precursors of mature ST. The culture was prepared, labelled and chased as described in Fig. 1; SDS-PAGE was, as in A and B, with phosphate buffer. The chase times are indicated above the photograph. The conversion of pre-pro-ST_A to pro-ST_A and the time-dependent disappearance of both species from the cells is clearly visible.

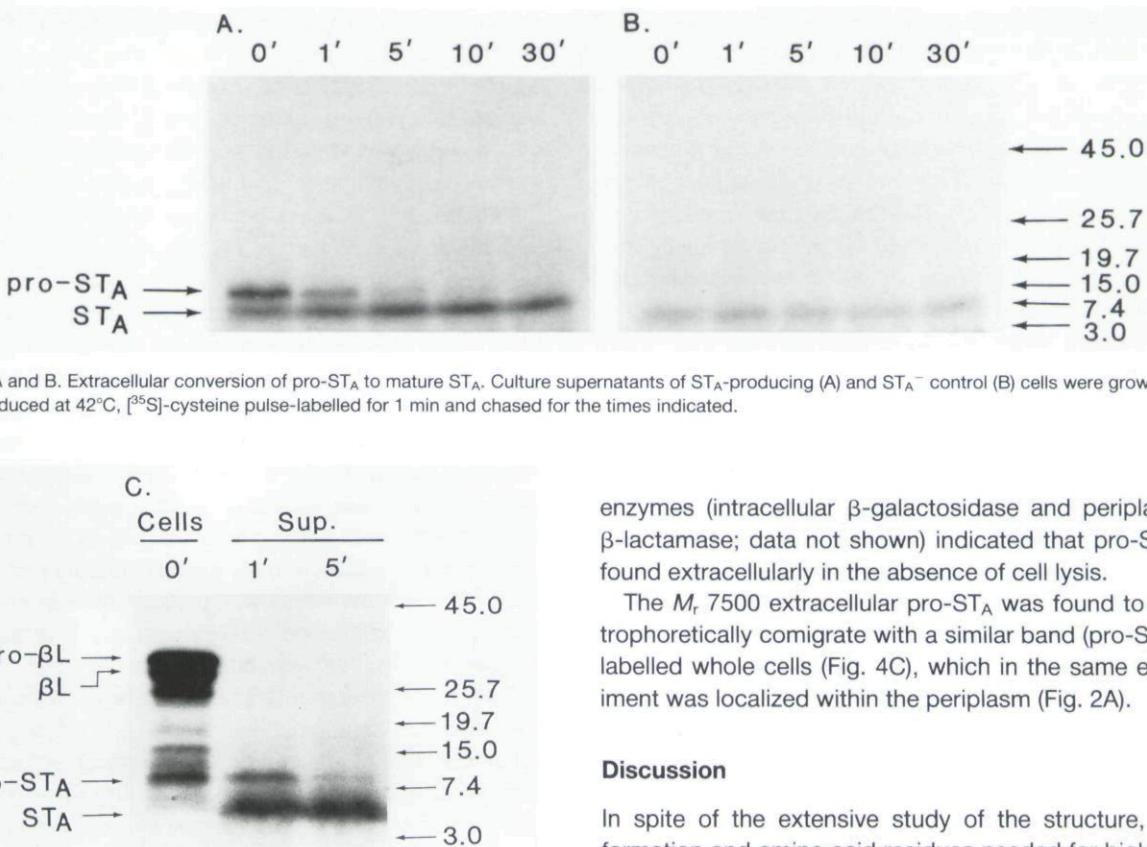


Fig. 4. A and B. Extracellular conversion of pro-ST_A to mature ST_A. Culture supernatants of ST_A-producing (A) and ST_A⁻ control (B) cells were grown at 29°C, induced at 42°C, [³⁵S]-cysteine pulse-labelled for 1 min and chased for the times indicated.

enzymes (intracellular β -galactosidase and periplasmic β -lactamase; data not shown) indicated that pro-ST_A is found extracellularly in the absence of cell lysis.

The M_r 7500 extracellular pro-ST_A was found to electrophoretically comigrate with a similar band (pro-ST_A) in labelled whole cells (Fig. 4C), which in the same experiment was localized within the periplasm (Fig. 2A).

Discussion

In spite of the extensive study of the structure, conformation and amino acid residues needed for biological activity of the mature ST_A (Houghten *et al.*, 1984; Yoshimura *et al.*, 1985; Gariepy *et al.*, 1986; Ohkubo *et al.*, 1986; Gariepy *et al.*, 1987), very little information has been obtained regarding the events that result in the delivery of ST_A to the exterior of the cell. In 1983, Guzmán-Verduzco *et al.* proposed that two independent proteolytic steps are required for the conversion of the 72-amino-acid precursor to the extracellular mature ST_A. The first cleavage between amino acids 19 and 20 of pre-pro-ST_A has since been substantiated by the sequencing of a cell-associated precursor of a fused pro-ST_A-LT_B polypeptide (Guzmán-Verduzco and Kupersztch, 1990, accompanying paper). Furthermore, a periplasmic location for an intermediate of ST_A was strongly supported by the detection of a thermo-activatable form of ST_A found in this compartment (Guzmán-Verduzco *et al.*, 1983).

Extracellular processing of pro-ST_A to mature ST_A

Figures 1B and 3C show that pro-ST_A is transiently cell-associated and specifically, that it is a periplasmic polypeptide that with time disappears from this compartment (Fig. 2A). This observation could be the result of its periplasmic conversion to ST_A; alternatively, pro-ST_A could pass through the outer membrane to become an extracellular polypeptide. To clarify these alternatives, pulse-chase experiments similar to the one shown in Fig. 3C were performed and the supernatant analysed. In Figure 4, it is shown that pro-ST_A was detected in the extracellular environment shortly after the pulse; upon chasing, pro-ST_A was converted to mature ST_A (M_r 4500) outside the cell (Fig. 4A). The extracellular processing of pro-ST_A to mature ST_A nears completion after 10 min (Fig. 4A). Bands with similar mobilities were not detected when the supernatants of the ST_A⁻ control strain were analysed in the same manner (Fig. 4B). Culture supernatants showed no β -lactamase outside the cell when pro-ST_A was most abundant. Additionally, proper localization of the marker

The data that we present indicate that the processing of ST_A may be different from that of other Gram-negative extracellular polypeptides. The detection of the intracellular species, pre-pro-ST_A (M_r 9800), whose export and processing is inhibited by the uncoupler CCCP (Fig. 3A, lane 3; Fig. 3B, lane 2) and of pro-ST_A, as both a periplasmic (Fig. 2A) and extracellular species (M_r 7500) (Fig. 4A), indicates that the toxin undergoes a two-step translocation process in order to reach the extracellular environment. The translocation through the inner membrane with cleavage of the amino-terminal signal

sequence may be analogous to what occurs initially in the export of many secreted peptides. Although the mechanism by which ST_A is transported through the outer membrane is not known, we had previously suggested that cleavage at the methionine (residue₅₃) that precedes the amino-terminal asparagine residue of the mature toxin occurs before its translocation to the exterior of the cell (Guzmán-Verduzco *et al.*, 1983; Guzmán-Verduzco and Kupersztoch, 1987). However, when we analysed the supernatant of labelled cells, pro-ST_A (M_r 7500) was found extracellularly where it was chased to mature ST_A (M_r 4500) (Fig. 4A). Thus, pro-ST_A, not mature ST, is the species which exits through the outer membrane. The intervening pro sequence between amino acids 20 and 53 does not contain a hydrophobic core but instead is rich in positively charged lysines (8 of 34 amino acids) and serines (6 out of 34) that could conceivably interact with the negative charges or hydrophilic region of outer membrane components. The interaction of this domain with components of the outer membrane is currently under investigation.

The initial difficulty of detecting the two cellular precursors of ST_A was due to the fact that the buffer we used initially in the SDS-PAGE system could not resolve pre-pro-ST_A from pro-ST_A (Fig. 1); this difficulty, as initially thought, did not result from the fact that the processing of pre-pro-ST_A to pro-ST_A was very fast, since both species were resolved when phosphate buffer was used instead of Tris buffer (Fig. 3), even when the cultures were not exposed to CCCP.

The theoretical and experimental sizes of pre-pro-ST_A, pro-ST_A and ST_A are 9800 and 7910 Daltons, 7500 and 5752 Daltons, and 4500 and 2048 Daltons, respectively. The differences between theoretical and experimental values could be accounted for by the unusual electrophoretic migration, caused by the high cysteine content, of the ST domain (Rasheed *et al.*, 1988). A similar effect was observed in the analysis of the fusion of ST_A to LT_B (Guzmán-Verduzco and Kupersztoch, 1987). The proposed cleavage sites are consistent with the facts that the mature ST_A with an apparent M_r of 4500 is indeed a 19-amino-acid peptide of the expected sequence (Yuan Yang and Y. M. Kupersztoch, unpublished) and that pro-ST_A-LT_B, a peptide that results from the fusion of estA to eltB, also has the expected sequence (Guzmán-Verduzco and Kupersztoch, 1990, accompanying paper).

It is possible that overproduction of a secreted peptide may affect its proper compartmentalization as well as the final localization of other exported polypeptides (Pages *et al.*, 1987; Click *et al.*, 1988). However, the appearance of pro-ST_A extracellularly occurs shortly after the pulse-chase. In addition, the culture supernatant containing pro-ST_A is otherwise free of periplasmic (β -lactamase, also hyperproduced) or cytoplasmic (β -galactosidase)

enzymes. Therefore, we feel that in the expression system we are using, the extracellular localization of pro-ST_A and its subsequent processing outside of the cell are part of the natural secretory pathway of ST_A. However, to conclusively show the natural secretory pathway, experiments must be performed using a low copy-number plasmid encoding estA3 under its natural conditions of expression.

Currently it remains unclear how soluble proteins destined for the periplasm can be distinguished from those targeted for the exterior of the cell. Also not understood is how, once in the periplasm, a peptide destined to be extracellular actually crosses the outer membrane, a relatively inert, rigid barrier which lacks a source of energy and proton-motive force. For some of these extracellular proteins, such as exotoxin A of *Pseudomonas aeruginosa*, a single proteolytic step has been shown to occur from precursor to mature toxin (Lory *et al.*, 1988). Presumably the polypeptide first interacts with the inner membrane, then through Bayer junctions or regions of fusion between the two membranes, it diffuses laterally to the outer membrane where it undergoes a conformational change which results in its release to the exterior of the cell. Different mechanisms of extracellular translocation are also seen with *E. coli*-expressed haemolysin (HlyA) and IgA protease from *Neisseria gonorrhoeae*. With the former peptide, there is no amino-terminal signal sequence but instead there is a carboxy-terminal sequence, essential for export, which interacts specifically with two hly gene products which have been proposed to form a pore through the membrane (Gray *et al.*, 1986). The *N. gonorrhoeae* IgA protease is synthesized as a 1532-amino-acid polypeptide and it has a 27-amino-acid amino-terminal signal sequence which is cleaved as the peptide is translocated through the inner membrane. Once in the periplasm, the carboxy-terminal 578-amino-acid amphipathic 'helper' domain is thought to direct the insertion of the polypeptide in the outer membrane, forming a pore for the excretion of the peptide (Pohlner *et al.*, 1987).

It has been suggested that because of the small size of ST_As, they would be able to leak out of the periplasm without a need for significant increase in outer membrane permeability or the intervention of a specific translocation system (Pugsley and Schwartz, 1985). Having shown that the last proteolytic step to yield mature ST_A is an extracellular event, the 53-amino-acid pro-ST_A is the species that traverses the outer membrane. While the outer membrane contains porins which allow free passage to hydrophilic molecules with little specificity, these channels of *E. coli* have an exclusion limit of 550–650 Daltons (Hancock and Nikaido, 1978); thus, it seems unlikely that pro-ST_A traverses the outer membrane through porins. While it is not known whether a specific translocation apparatus

exists, chromosomal mutations that affect extracellular delivery of a heat-stable enterotoxin of *E. coli* have been described. However, since the wild-type toxin used in these studies was not biologically active in the suckling-mouse assay, a property common to all ST_As, it seems unlikely that the enterotoxin affected was the methanol-soluble ST; it was probably ST_B instead (Sack, 1975; Silva *et al.*, 1978). Nevertheless, once outside the cell, the pro-ST_A is converted to mature toxin. It is known that *E. coli* possess a variety of surface-exposed, membrane-bound, proteolytic enzymes (Sugimura and Higashi, 1988). The inability of the supernatant of labelled spheroplasts to convert pro-ST_A to ST_A (Fig. 2C) is contrasted by the maturation seen outside intact cells (Fig. 4A); these observations may suggest that outer membrane components, disrupted during the spheroplasting procedure, or extracellular proteases may be important during ST_A maturation. We are currently investigating whether this conversion is a self-catalytic event or whether there are other proteases involved.

In an earlier report (Lathe *et al.*, 1980), it was shown that incubation of ST_A-encoding DNA in an *in vitro* transcription/translation system results in the appearance of biologically active enterotoxin. Two polypeptide species were detected: an *M_r* 10000 and an *M_r* 7000 protein. In view of the data that we present here and information presented elsewhere (Rasheed *et al.*, 1988), it is possible that the *M_r* 10000 species is pre-pro-ST_A and the *M_r* 7000 species is pro-ST_A. This would mean that either or both molecules are enterotoxicogenic or, alternatively, that mature ST_A was produced in the incubation mixture. This would be the case if the conversion of pro-ST_A to ST_A is a self-catalytic event or if proteases were present in the extract.

Studies to clearly define the characteristics of the precursor forms of ST_A and to determine the nature of the extracellular cleavage of pro-ST_A are in progress.

Experimental procedures

Bacterial strains, plasmids and media

E. coli strain RR1 (Bolivar *et al.*, 1977), a recA⁺ derivative of HB101 (*F*⁻, *hsds20*, *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20*, *xyl-5*, *mtl-1*, *supE44*, λ^-), was used in these experiments. Plasmids pGP1-2 and pT7-3 (Tabor and Richardson, 1985) were kindly supplied by S. Tabor. Plasmid pYK151 was constructed by inserting a 960 *Bam*H I *estA3* fragment into plasmid pT7-3 digested with the same enzyme. In this plasmid, *estA3* is downstream from the T7 promoter of plasmid pT7-3 and is followed, in the same orientation, by the β -lactamase gene. In all radiolabelling experiments, cells were grown in C-broth (Guzmán-Verduzco and Kupersztch, 1987) of the following composition: 2 g NH₄Cl, 6 g Na₂HPO₄, 3 g KH₂PO₄, 3 g NaCl, 0.018 g MgCl₂, 6 g yeast extract and 3% glycerol per litre of solution. C-medium was supplemented, when indicated, with ampicillin (50 μ g ml⁻¹), kanamycin (50 μ g ml⁻¹), thiamine (0.5 μ M), L-proline (200 μ g ml⁻¹) and D,L-leucine (100 μ g ml⁻¹).

Pulse-labelling of whole cells and spheroplasts

Strains RR1 (pGP1-2) (pT7-3) and RR1 (pGP1-2) (pYK151) were grown separately at 29°C in supplemented C-broth to an OD₅₉₀ of 0.8. The cells were harvested by centrifugation and resuspended in 30% of the original volume of the same broth pre-warmed to 29°C. The cultures were then shifted to 42°C for 15 min, rifampicin (200 μ g ml⁻¹) was added, and the cultures incubated for 10 min at 42°C after which they were shifted back to 29°C and incubated for 15 min more. When indicated, after the 42°C incubations, 50 μ M CCCP (final concentration) dissolved in dimethyl sulphoxide was added and then the cultures were shifted down to 29°C for 15 min. The cultures were labelled with 15 μ Ci ml⁻¹ of radioactive [³⁵S]-cysteine (specific activity of 1000 Ci mmol⁻¹) for the times indicated at 29°C. Pre-warmed supplemented C-broth containing an excess of unlabelled cysteine (8 mg ml⁻¹) was then added and incubation continued for the indicated chase times. Aliquots of labelled cultures taken before and during the chase were added to one half volume of 4°C stop solution (0.04% chloramphenicol, 0.4 M sodium azide and 0.02 M 2,4-dinitrophenol). If cells were to be subsequently spheroplastral, this stop solution also contained 0.25 M sucrose. Cells were separated from supernatant by centrifugation; to obtain whole-cell lysates of cells, the pellet was immediately resuspended in lysis buffer (60 mM Tris-HCl pH 7.6, 2% SDS, 10% glycerol, 0.01% bromophenol blue and either 5% 2-mercaptoethanol or 100 mM dithiothreitol when indicated) and stored at -20°C until analysed by SDS-PAGE. Before analysis, all lysates were thawed and boiled for 5 min.

Cells were converted to spheroplasts by the procedure of Witholt (1976), modified as follows: all steps were performed at 4°C, the mild osmotic shock by the addition of water was eliminated and the cellular suspension contained, when indicated, either 0.04% chloramphenicol and 0.4 M sodium azide or 50 μ M CCCP. Cell density was adjusted to 5–6 mg of cell dry mass per millilitre. When spheroplastral was complete as judged by phase microscopy, cells and supernatant (periplasmic fraction) were separated by centrifugation. The spheroplasts were either lysed or used in pulse-chase experiments and then lysed. To pulse-label spheroplasts, they were obtained from 100 ml cultures and resuspended in 1 ml of C-broth containing 0.25 M sucrose and 0.02 M MgCl₂. After radiolabelling, the spheroplasts and supernatants were separated by centrifugation and the spheroplasts were resuspended in an equal volume of stop solution. When they were to be lysed, they were resuspended in an equal volume of 200 mM Tris-HCl (pH 8) supplemented with equivalent concentrations of reagents contained in the periplasmic fraction. The spheroplast suspension, either in Tris-HCl or in stop solution, was submerged in an ice bath and lysed by brief sonication by 10-s pulses with 40-s intervals using a model MW-350 Sonifier (Branson Sonic Power Co., Danbury, CT), equipped with a special stepped microtip model 420 (Heat Systems Ultrasonics, Farmingdale, NY). Sonic extracts were cleared by low-speed centrifugation (1200 \times g for 8 min).

After the assay of periplasmic β -lactamase (O'Callaghan *et al.*, 1972) and intracellular β -galactosidase (Miller, 1972), both the periplasm and the lysed spheroplast fractions were reduced with 1% 2-mercaptoethanol at 25°C for 30 min followed by 10% TCA precipitation for 20 min in an ice bath. Pellets were collected, washed with ethanol, neutralized with 5 N sodium hydroxide, dissolved in lysis buffer and boiled for 5 min prior to electrophoresis and fluorography.

The samples analysed by SDS-PAGE in phosphate buffer were first reduced with DTT and then carboxyamidated with iodoacetamide as follows. The samples were suspended in 1 M Tris-HCl (pH 8), 80 mM DTT, 2% SDS and 2 mM EDTA. In some cases, resuspension of the cells was aided by brief sonication. The samples along with periplasmic fractions which had been supplemented with 80 mM DTT and 2% SDS were each boiled for 5 min followed by an incubation at 37°C for 20 min. The extracts were boiled for 3 min before the addition of a 10% molar excess (over the amount of reducing agent) of iodoacetamide followed by incubation for 30 min at 25°C in the dark. Each was then precipitated with 5 volumes of acetone at -20°C for 30 min. Pellets were dissolved in lysis buffer (0.010 M phosphate buffer pH 7.1, 1% SDS, 10% glycerol, 0.25% bromophenol blue and 5% 2-mercaptoethanol) and boiled for 5 min before electrophoresis and fluorography.

Preparation of culture supernatant for electrophoresis

Either 1% 2-mercaptoethanol or 50 mM dithiothreitol was added to the culture supernatant, which was then incubated at 25°C for 30 min; one half of the volume of the supernatant was supplemented with unlabelled *estA3⁻* cells equivalent to the original culture and precipitated for 20 min at 4°C with an equal volume of cold 20% TCA. The neutralized pellets were dissolved in lysis buffer. The other half of the supernatant was applied to a C-18 reverse-phase chromatography cartridge (Sep-pak, Waters Associates) (D. C. Robertson, personal communication), pre-wet and pre-washed with 5 ml of 90% methanol in 10 mM ammonium acetate, pH 5.7 and with 10 ml of 10 mM ammonium acetate pH 5.7, respectively. Following the application of the sample, the cartridge was washed with 10 ml of 10 mM ammonium acetate, pH 5.7, followed by 30% methanol in the same buffer. ST_A was eluted with 3 ml of 60% methanol in the same buffer. When necessary, rifampicin was removed from the solution by extracting it with an equal volume of chloroform. The aqueous phase was removed, dried in a speed-vac concentrator, and dissolved in lysis buffer.

Electrophoresis

Peptides were separated by SDS-PAGE using 0.1% SDS, with either a 20% linear (Laemmli, 1970) or a 10–15% gradient polyacrylamide gel containing a 10:1 ratio of acrylamide to *N,N'*:diallyltartardiamide. The 15% acrylamide solution contained 75% glycerol (Rasheed *et al.*, 1988). Electrophoresis was performed at 4°C at 25 mAmp in a Protean II slab gel chamber (Biorad, California) or at 4°C at 200 volts in a Mini-Protean II dual slab cell.

The separation of the cellular precursor of ST was accomplished by SDS-PAGE in an 18% polyacrylamide gel (Laemmli, 1970) containing a 10:1 ratio of *N,N'*:diallyltartardiamide in 0.1 M phosphate buffer (pH 7.1) (Inouye and Guthrie, 1969). Thus, the separating gel contained 0.1 M phosphate buffer (pH 7.1) 0.1% SDS, 0.05% ammonium persulphate and 0.05% TEMED. The 4% stacking gel also contained 0.1% SDS and 0.1 M phosphate buffer (pH 7.1). Electrophoresis was at 46 volts at 25°C for 22 h in 0.1 M NaH₂PO₄ (pH 7.1) and 0.5% SDS.

The *M_r* of pre-stained molecular weight markers (BRL, Maryland) was determined under the different electrophoretic conditions in relation to the known molecular size of the unstained

species. After electrophoresis, the gels were stained with Coomassie brilliant blue R250 and the *M_r* established. Subsequently, only the stained markers were included and the *M_r* values of ST-related polypeptides were determined in reference to them.

After electrophoresis of [³⁵S]-cysteine-radiolabelled polypeptides, the gels were not fixed but were directly prepared for fluorography using sodium salicylate (Chamberlain, 1979). The gels were dried before exposure to pre-flashed (Laskey and Mills, 1975) X-omat film.

Acknowledgements

We thank Stanley Tabor for supplying plasmids pGP1-2 and pT7-3, and Frances E. Powell and Frances K. Tachias for technical assistance. We also thank Leon Eidels and Heather Stieglitz for the critical review of the manuscript, and Cindy Baselski for typing it. The work was supported in part by Public Health Service grant AI21698 from the National Institutes of Health.

References

- Aimoto, S., Takao, T., Shimonishi, Y., Hara, S., Takeda, T., Takeda, Y., and Miwatani, T. (1982) Amino-acid sequence of a heat-stable enterotoxin produced by human enterotoxigenic *Escherichia coli*. *Eur J Biochem* **129**: 257–263.
- Betley, M.J., Miller, V.L., and Mekalanos, J.J. (1986) Genetics of bacterial enterotoxins. *Annu Rev Microbiol* **40**: 577–605.
- Bolivar, G., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heynecker, H.L., Boyer, H.W., Crosa, J.H., and Falkow, S. (1977) Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**: 95–113.
- Burgess, M.N., Bywater, R.J., Cowley, C.M., Mullan, N.A., and Newsome, P.M. (1978) Biological evaluation of a methanol-soluble, heat-stable *Escherichia coli* enterotoxin in infant mice, pigs, rabbits, and calves. *Infect Immun* **21**: 526–531.
- Chamberlain, J.P. (1979) Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. *Analyt Biochem* **98**: 132–135.
- Click, E.M., McDonald, G.A., and Schnaitman, C.A. (1988) Translational control of exported proteins that results from OmpC porin over-expression. *J Bacteriol* **170**: 2005–2011.
- Daniels, C.J., Bole, D.G., Quay, S.C., and Oxender, D.L. (1981) Role for membrane potential in the secretion of protein into the periplasm of *Escherichia coli*. *Proc Natl Acad Sci USA* **78**: 5396–5400.
- Gariépy, J., Lane, A., Frayman, F., Wilbur, D., Robien, W., Schoolnik, G.K., and Jardetzky, O. (1986) Structure of the toxic domain of the *Escherichia coli* heat-stable enterotoxin STI. *Biochemistry* **25**: 7854–7866.
- Gariépy, J., Judd, A.K., and Schoolnik, G.K. (1987) Importance of disulfide bridges in the structure and activity of *Escherichia coli* enterotoxin STI_b. *Proc Natl Acad Sci USA* **84**: 8907–8911.
- Gray, L., Mackman, N., Nicaud, J.-M., and Holland, I.B. (1986) The carboxy-terminal region of haemolysin 2001 is required for secretion of the toxin from *Escherichia coli*. *Mol Gen Genet* **205**: 127–133.
- Guzmán-Verduzco, L.M., and Kupersztoch, Y.M. (1987) Fusion of *Escherichia coli* heat-stable enterotoxin and heat-labile enterotoxin B subunit. *J Bacteriol* **169**: 5201–5208.
- Guzmán-Verduzco, L.M., and Kupersztoch, Y.M. (1989) Rectification of two *Escherichia coli* heat-stable enterotoxin allele

- sequences and lack of biological effect of changing the carboxy-terminal tyrosine to histidine. *Infect Immun* **57**: 645–648.
- Guzmán-Verduzco, L.M., and Kupersztoch, Y.M. (1990) Export and processing analysis of a fusion between the extracellular heat-stable enterotoxin and the periplasmic B subunit of the heat-labile enterotoxin in *Escherichia coli*. *Mol Microbiol* **4**: 253–264.
- Guzmán-Verduzco, L.M., Fonseca, R., and Kupersztoch-Portnoy, Y.M. (1983) Thermoactivation of a periplasmic heat-stable enterotoxin of *Escherichia coli*. *J Bacteriol* **154**: 146–151.
- Hancock, R.E.W., and Nikaido, H. (1978) Outer membranes of gram-negative bacteria. XIX. Isolation from *Pseudomonas aeruginosa* PAO1 and use in reconstitution and definition of the permeability barrier. *J Bacteriol* **136**: 381–390.
- Hayashi, S., and Wu, H.C. (1985) Accumulation of prolipoprotein in *Escherichia coli* mutants defective in protein secretion. *J Bacteriol* **161**: 949–954.
- Houghten, R.A., Ostresh, J.M., and Klipstein, F.A. (1984) Chemical synthesis of an octadecapeptide with the biological and immunological properties of human heat-stable *Escherichia coli* enterotoxin. *Eur J Biochem* **145**: 157–162.
- Inouye, M., and Guthrie, J.P. (1969) A mutation which changes a membrane protein of *E. coli*. *Proc Natl Acad Sci USA* **64**: 957–961.
- Ito, K. (1982) Purification of the precursor form of maltose-binding protein, a periplasmic protein of *Escherichia coli*. *J Biol Chem* **257**: 9895–9897.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.
- Laskey, R.A., and Mills, A.D. (1975) Quantitative film detection of ³H and ¹⁴C in polyacrylamide gels by fluorography. *Eur J Biochem* **56**: 335–341.
- Lathe, R., Hirth, P., DeWilde, M., Harford, N., and Lecocq, J.-P. (1980) Cell-free synthesis of enterotoxin of *E. coli* from a cloned gene. *Nature* **284**: 473–474.
- Lazure, C., Seidah, N.G., Chretien, M., Lallier, R., and St.-Pierre, S. (1983) Primary structure determination of *Escherichia coli* heat-stable enterotoxin of porcine origin. *Can J Biochem Cell Biol* **61**: 287–292.
- Lory, S., Strom, M.S., and Johnson, K. (1988) Expression and secretion of the cloned *Pseudomonas aeruginosa* exotoxin A by *Escherichia coli*. *J Bacteriol* **170**: 714–719.
- Miller, J.H. (1972) In *Experiments in Molecular Genetics*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, pp. 412–414.
- Moseley, S.L., Hardy, J.W., Huq, M.I., Echeverria, P., and Falkow, S. (1983) Isolation and nucleotide sequence determination of a gene encoding a heat-stable enterotoxin of *Escherichia coli*. *Infect Immun* **39**: 1167–1174.
- O'Callaghan, C.H., Morris, A., Kirby, S.M., and Shingler, A.H. (1972) Novel method for detection of β-lactamase by using a chromogenic cephalosporin substrate. *Antimicrob Agents Chemother* **1**: 283–288.
- Ohkubo, T., Kobayashi, Y., Shimonishi, Y., and Kyogoku, Y. (1986) A conformational study of polypeptides in solution by ¹H-nmr and distance geometry. *Biopolymers* **25**: S123–S134.
- Pages, J.-M., Anba, J., and Lazdunski, C. (1987) Conditions leading to secretion of a normally periplasmic protein in *Escherichia coli*. *J Bacteriol* **169**: 1386–1390.
- Pohlner, J., Halter, R., Beyreuther, K., and Meyer, T.F. (1987) Gene structure and extracellular secretion of *Neisseria gonorrhoeae* IgA protease. *Nature* **325**: 458–462.
- Pugsley, A.P., and Schwartz, M. (1985) Export and secretion of proteins by bacteria. *FEMS Microbiol Rev* **32**: 3–38.
- Rasheed, J.K., Guzmán-Verduzco, L.M., and Kupersztoch, Y.M. (1988) Hyperproduction of heat-stable enterotoxin (ST_{A4}) of *Escherichia coli* and analysis of the unusual electrophoretic behavior of reduced and alkylated forms of ST_{A5}. *Microb Pathogen* **5**: 333–343.
- Ronnberg, B., Wadstrom, T., and Jornvall, H.T. (1983) Structure of a heat-stable enterotoxin produced by a human strain of *Escherichia coli*. *FEBS Lett* **155**: 183–186.
- Sack, R.B. (1975) Human diarrheal disease caused by enterotoxigenic *Escherichia coli*. *Annu Rev Microbiol* **29**: 333–353.
- Saeed, A.M.K., Magnuson, N.S., Sriranganathan, N., Burger, D., and Cosand, W. (1984) Molecular homogeneity of heat-stable enterotoxins produced by bovine enterotoxigenic *Escherichia coli*. *Infect Immun* **45**: 242–247.
- Sekizaki, T., Akashi, H., and Terakado, N. (1985) Nucleotide sequences of the genes for *Escherichia coli* heat-stable enterotoxin I of bovine, avian, and porcine origins. *Am J Vet Res* **46**: 909–912.
- Silva, M.L.M., Maas, W.K., and Gyles, C.L. (1978) Isolation and characterization of enterotoxin-deficient mutants of *Escherichia coli*. *Proc Natl Acad Sci USA* **75**: 1384–1388.
- So, M., and McCarthy, B.J. (1980) Nucleotide sequence of the bacterial transposon Tn1681 encoding a heat-stable (ST) toxin and its identification in enterotoxigenic *Escherichia coli* strains. *Proc Natl Acad Sci USA* **77**: 4011–4015.
- Stieglitz, H., Cervantes, L., Robledo, R., Fonseca, R., Covarrubias, L., Bolívar, R., and Kupersztoch, Y.M. (1988) Cloning, sequencing, and expression in ficol-generated minicells of an *Escherichia coli* heat-stable enterotoxin gene. *Plasmid* **20**: 42–53.
- Sugimura, K., and Higashi, N. (1988) A novel outer-membrane-associated protease in *Escherichia coli*. *J Bacteriol* **170**: 3650–3654.
- Tabor, S., and Richardson, C.C. (1985) A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci USA* **82**: 1074–1078.
- Takao, T., Hitouji, T., Aimoto, S., Shimonishi, Y., Hara, S., Takeda, T., Takeda, Y., and Miwatani, T. (1983) Amino acid sequence of a heat-stable enterotoxin isolated from enterotoxigenic *Escherichia coli* strain 18D. *FEBS Lett* **152**: 1–5.
- Thompson, M.R., and Giannella, R.A. (1985) Revised amino acid sequence for a heat-stable enterotoxin produced by an *Escherichia coli* strain (18D) that is pathogenic for humans. *Infect Immun* **47**: 834–836.
- von Heijne, G. (1985) Signal sequences. The limits of variation. *J Mol Biol* **184**: 99–105.
- Whipp, S.C., Moon, H.W., and Argenzio, R.A. (1981) Comparison of enterotoxic activities of heat-stable enterotoxins from class 1 and class 2 *Escherichia coli* of swine origin. *Infect Immun* **31**: 245–251.
- Wickner, W.T., and Lodish, H.F. (1985) Multiple mechanisms of protein insertion into and across membranes. *Science* **230**: 400–407.
- Witholt, B., Boekhout, M., Brock, M., Kingma, J., van Herikhuizen, H., and de Leij, L. (1976) An efficient and reproducible procedure for the formation of spheroplasts from variously grown *Escherichia coli*. *Analyt Biochem* **74**: 160–170.
- Yoshimura, S., Ikemura, H., Watanabe, H., Aimoto, S., Shimonishi, Y., Hara, S., Takeda, T., Miwatani, T., and Takeda, Y. (1985) Essential structure for full enterotoxigenic activity of heat-stable enterotoxin produced by enterotoxigenic *Escherichia coli*. *FEBS Lett* **181**: 138–142.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.