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Synthesis of *Escherichia coli* Heat-Stable Enterotoxin STp as a Pre-Pro Form and Role of the Pro Sequence in Secretion

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***Escherichia coli* heat-stable enterotoxin STp is presumed from its DNA sequence to be synthesized in vivo as a 72-amino-acid residue precursor that is cleaved to generate mature STp consisting of the 18 carboxy-terminal amino acid residues. There are two methionine residues in the inferred STp sequence in addition to the methionine residue at position 1. In order to confirm production of the STp 72-amino-acid residue precursor, we substituted the additional methionine residues by oligonucleotide-directed site-specific mutagenesis. Since these substitutions did not cause a significant change in STp production, it can be concluded that STp is normally synthesized as the 72-amino-acid residue precursor. The length of the STp precursor indicates the existence of a pro sequence between the signal peptide and the mature protein. In order to identify the pro sequence and determine its role in protein secretion, deletion and fusion proteins were made. A deletion mutant in which the gene fragment encoding amino acid residues 22 to 53 of STp was removed was made. STp activity was found in the culture supernatant of cells. Amino acid sequence analysis of the purified STp deletion mutant revealed that the pro sequence encompasses amino acid residues 20 to 54. A hybrid protein consisting of STp amino acids 1 to 53 fused in frame from residue 53 to nuclease A was not secreted into the culture supernatant. These results indicate that the pro sequence does not function to guide periplasmic protein into the extracellular milieu.**

The heat-stable enterotoxins of *Escherichia coli* (STs) have been classified into two groups according to their physiological and biological properties (1). One is STa (also referred to as ST I), and the other is STb (also referred to as ST II). STa is ethanol soluble and active in the suckling mouse model and in the porcine ligated ileum model. The molecular structure of STa has been determined (3, 21). In contrast, STb is methanol insoluble and is positive only in the weaned-pig ligated ileum model. The molecular structure of STb has not been determined.

STa falls further into two classes. The first is composed of 18 amino acid residues, and the second is composed of 19 amino acid residues. The first toxin is designated STp and the second is designated STh, since they originate from porcine and human strains of enterotoxigenic *E. coli*, respectively. Both STas share an almost identical carboxy-terminal sequence (23, 26). Four STa genes (one STp gene and three STh genes) have been cloned, and the nucleotide sequences have been determined (6, 23, 24). Sequencing revealed that two of the three STh genes are identical and that the nucleotide sequence of the remaining STh gene is very similar to that of the two identical STh genes. However, the differences between the STp gene and the STh genes are great (6, 23). Despite the nucleotide sequence divergence observed among these genes, they are predicted to encode peptides of the same length, 72 amino acid residues (Fig. 1). This indicates that all STs are synthesized in vivo as large precursors which then undergo extensive processing. However, little is known about the predicted ST precursors, particularly that of STp which has not been studied to date. Furthermore, because the inferred amino acid sequences of STp and STh are considerably different, it is possible that they are synthesized, processed, and secreted in different ways (Fig. 1). In this study, we examined the synthesis and secretion of STp by using gene manipulations to determine the existence and function of the STp precursor.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* HB101 was used as the host strain in all experiments and was cultured in Luria broth (14). Plasmid Tc-1, which carries the *E. coli* STp gene, was kindly provided by M. So. Plasmid Tc-1, approximately 4.7 kilobase pairs in length, contains the 350-base-pair *Taq*I-*Hin*fI fragment of Tn*1681* at the *Hin*dIII site (23). Plasmid pIN-III-*Omp*A-#98 was used as the source of the staphylococcal nuclease A gene. The plasmid has an extra sequence of 33 base pairs between the coding sequences for the *Omp*A signal peptide and nuclease A, and there are unique *Eco*RI and *Bam*HI sites in the extra sequence (25).

Plasmid pIN-I-A3 is one of the high-level expression cloning vectors developed by the laboratory of Inouye (12). The *E. coli* lipoprotein promoter is utilized to express foreign genes cloned into the vector, and there are unique *Eco*RI, *Hin*dIII, and *Bam*HI sites immediately after the translational initiation codon to facilitate insertion of foreign genes.

Oligonucleotide-directed site-specific mutagenesis. Oligonucleotide-directed site-specific mutagenesis was performed to substitute amino acid residues of STp by using the plasmid method described by Inouye and Inouye (8). The plasmid used for the mutagenesis was Tc-1. Mutagenic oligonucleotides were synthesized and purified as described previously (16). Oligonucleotide 601 (16-mer; AGAAAATTCGAA CAAC) was designed to direct the mutagenesis of STp precursor amino acid residues Met-53 to Ser-53 and to introduce a *Nsp*V site (Fig. 1; numbers above the amino acid residues indicate the positions of each residue in the partic-

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            1   2   3   4   5   6   7   8   9   10  11  12  13
STp(STA1)   Met-Lys-Lys-Leu-Met-Leu-Ala-Ile-Phe-Ile-Ser-Val-Leu-
STh(STA2)               Ser Ile     Phe         Leu
STh(STA3)               Ser Ile     Phe         Leu

            14  15  16  17  18  19  20  21  22  23  24  25  26
STp(STA1)   Ser-Phe-Pro-Ser-Phe-Ser-Gln-Ser-Thr-Glu-Ser-Leu-Asp-
STh(STA2)           Ser Pro     Ala     Asp Ala Lys Pro Ala Gly
STh(STA3)           Ser Pro     Ala     Asp Ala Lys Pro Val Glu

            27  28  29  30  31  32  33  34  35  36  37  38  39
STp(STA1)   Ser-Ser-Lys-Glu-Lys-Ile-Thr-Leu-Glu-Thr-Lys-Lys-Cys-
STh(STA2)                                       Ser
STh(STA3)                                       Ser

            40  41  42  43  44  45  46  47  48  49  50  51  52
STp(STA1)   Asp-Val-Val-Lys-Asn-Asn-Ser-Glu-Lys-Lys-Ser-Glu-Asn-
STh(STA2)   Asn Ile Val     Lys Asn Asn Glu Ser Ser Pro     Ser
STh(STA3)   Asn Ile Ala     Lys Ser Asn Lys Ser Gly Pro     Ser

            53  54  55  56  57  58  59  60  61  62  63  64  65
STp(STA1)   Met-Asn-Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-
STp(STA2)           Ser Ser Asn
STp(STA3)           Ser Ser Asn

            66  67  68  69  70  71  72
STp(STA1)   Pro-Ala-Cys-Ala-Gly-Cys-Tyr
STh(STA2)               Thr
STh(STA3)               Thr
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FIG. 1. Comparison of the amino acid sequences of STp and STh inferred from their nucleotide sequences. The STp(STA1) sequence is from So and McCarthy (23), and STh(STA2) and STh(STA3) are from Guzman-Verduzco and Kupersztoch (6). For the STh(STA2) and STh(STA3) sequences, only the amino acids that differ from STp(STA1) are shown. Empty spaces indicate identical amino acids.

ular ST sequence). Oligonucleotide 602 (22-mer; ATGAA AAATCTAGAGTTGGCAA) was designed to direct the mutagenesis of STp precursor amino acid residues Lys-3, Leu-4, Met-5 to Asn-3, Leu-4, and Glu-5 and to introduce a *Xba*I site.

Deletion mutant. The STp gene encoding amino acids 22 to 53 was deleted from pKK601 as follows. The plasmid has one *Nsp*V site and three *Hin*cII sites. One *Hin*cII site is in the STp gene, and the others are in the drug resistance genes originated from pBR322 (23). Plasmid pKK601 was digested with *Nsp*V and *Hin*cII (partially), and the resultant linear 4.6-kilobase fragments were purified. The fragments were treated with the Klenow fragment of DNA polymerase I (Klenow) in the presence of four deoxyribonucleotides (four deoxynucleoside triphosphates) to fill in the 5′ single-stranded regions. *Bam*HI linkers (CGGATCCG) were ligated to the filled-in fragment by using T4 DNA ligase. The transformants were selected by ampicillin resistance, and the proper construction was confirmed by DNA sequencing. The resulting plasmid was designated pKK603 (Fig. 2). The predicted and confirmed partial amino acid sequence of pKK603-encoded STp is shown in Fig. 2. The numbers in Fig. 2 indicate the positions of the corresponding amino acids in the sequence of pKK601-encoded STp. The DNA sequence encoding STp amino acid residues 22 to 53 was deleted from pKK601, and the linker peptide consisting of Gly, Ser, and Ala was inserted into pKK603-encoded STp (Fig. 2).

ST activity of cell. *E. coli* HB101 cells harboring appropriate plasmids were cultured in Luria broth containing ampicillin (50 μg/ml) to an optical density of 0.4 at 600 nm. The culture supernatants were obtained by centrifugation at 10,000 × *g*. The cell pellets were washed and suspended in 0.1 volume of phosphate-buffered saline. The cell extracts were prepared by sonication of the cell pellets. The protein concentrations of all cell extracts were adjusted to 4.15 mg/ml. ST activities of the culture supernatants and the cell extracts were examined in the suckling mouse assay. Protein

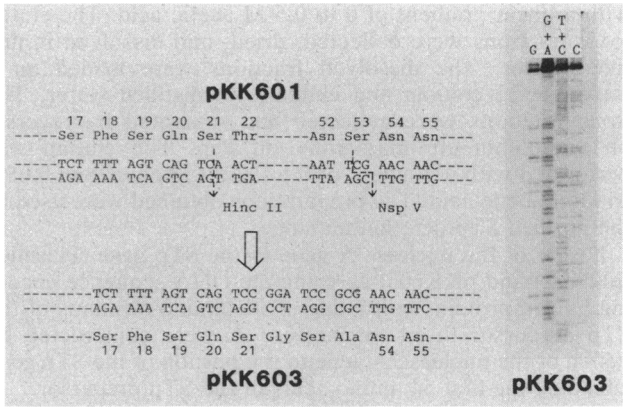

FIG. 2. Deletion of the pro sequence of STp and the nucleotide sequence of the STp deletion mutant. pKK601 was obtained from Tc-1 (the plasmid containing the wild-type gene) by using oligonucleotide-directed site-specific mutagenesis to substitute serine for methionine at position 53 of STp and introduce an *Nsp*V site. The deletion of the STp gene encoding the pro sequence from pKK601 was performed as described in Materials and Methods. The plasmid obtained was designated pKK603. The nucleotide sequences around the STp gene of pKK603 were determined by the method of Maxam and Gilbert (13) as follows. The plasmid DNA was digested with *Hin*dIII and labeled with [α-^{32}P]dATP by Klenow. The labeled DNA was then cleaved with *Alu*I. The fragments carrying the STp structural gene were purified and sequenced by the method of Maxam and Gilbert (13), and the chemical cleavage products were electrophoretically separated on a denaturing 10% polyacrylamide gel. The dashed lines and arrows indicate the sites recognized by the restriction endonucleases. The open arrow indicates that pKK603 is derived from pKK601.

concentration was determined by the method of Lowry et al. (11).

Purification of the STp deletion mutant protein. STp produced by *E. coli* HB101(pKK603) (STp deletion mutant) was purified from the culture supernatant. The cells were grown in Luria broth containing ampicillin (50 μg/ml) at 37°C overnight with shaking. The supernatant was separated from the cells by centrifugation. STp was precipitated from the supernatant by ammonium sulfate (60 g/100 ml), and the resulting precipitate was recovered by centrifugation and then dissolved in distilled water. This preparation was then dialyzed against distilled water with membrane tubing (Spectra/Por 6; Spectrum Medical Industries, Inc., Los Angeles, Calif.).

The crude preparation of the STp deletion mutant was further purified by successive column chromatographies on DEAE-Sephacel, DEAE-Sephadex A-25, Mono Q, Superose 12, and Pep (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden). The crude preparations of STp were applied to DEAE-Sephacel columns equilibrated with distilled water. Proteins were eluted with a gradient of 0 to 1 M acetic acid. The fractions containing STp activity were collected and concentrated by rotary evaporator under a vacuum. The materials thus obtained were dissolved in distilled water and applied to DEAE-Sephadex A-25 columns. After the columns were washed with distilled water, the absorbed materials were eluted with a gradient of 0 to 0.5 M acetic acid, and the toxic fractions were collected, dried, and dissolved in distilled water. The materials obtained were further purified by successive column chromatographies on Mono Q, Superose 12, and Pep by the fast protein liquid chromatography system (Pharmacia). The Mono Q column was developed

with a linear gradient of 0 to 0.5 M acetic acid. The eluted toxic fractions were collected, dried, and dissolved in distilled water. The dissolved fractions were loaded on a Superose 12 column and eluted with distilled water. The toxic fractions were collected and separated by reverse-phase column chromatograhy on Pep. The elution was performed with a gradient of 0 to 50% acetonitrile in 0.05% trifluoroacetic acid. The preparations obtained were used as the purified STp deletion mutant.

Fusion of the nuclease A gene to the STp gene. Plasmids (pKK604 and pKK605) carrying the DNA sequence encoding hybrid proteins consisting of the amino terminus of the STp precursor fused to nuclease A were constructed by fusion of the nuclease A gene to the portion of the STp gene encoding the first 54 amino acids of the STp precursor.

The construction of pKK604 was performed as follows. A fragment of about 1,300 base pairs encoding the nuclease A gene was obtained by *Eco*RI-*Sal*I digestion of pIN-III-*Omp*A-#98 and was treated with Klenow to fill in both 5′ single-stranded regions. The treated fragment was ligated with the Klenow-treated 4.6-kilobase *Hin*cII-*Nsp*V fragment of pKK601 which is described above. The resulting plasmids were transformed into competent HB101 cells. The transformants were plated onto toluidine blue-DNA agar plates (22; Nissui Co., Tokyo, Japan) containing ampicillin (50 μg/ml). The ampicillin-resistant transformants were selected for nuclease activity, which showed up as pink colonies on the DNA agar plates, and plasmids were isolated from these transformants.

In order to construct pKK605, pIN-III-*Omp*A-#98 was digested with *Bam*HI and *Sal*I, and the resulting ca. 1,300-base-pair fragment encoding the nuclease A gene was isolated and treated with Klenow to fill in 5′ single-stranded regions. The treated fragment was cloned into the Klenow-treated *Nsp*V site of pKK601 by blunt-end ligation. The reaction mixture was transformed into competent HB101 cells, and the transformants were selected on ampicillin-DNA agar plates for nuclease activity as described above.

Fusion of nuclease A gene to pIN-I-A3. To make the STp-nuclease A hybrid protein, the coding region for nuclease A was ligated to the *Bam*HI site of pIN-I-A3 as described below, and the resulting plasmid was designated pKK606.

Plasmid pIN-I-A3 was digested with *Bam*HI, and the resulting linear fragment was isolated. The fragment was ligated to the *Bam*HI-*Sal*I fragment of pIN-III-*Omp*A-#98 encoding the nuclease gene. The resulting fragments were treated with Klenow to fill in their 5′ single-stranded regions and then circularized by incubation with T4 DNA ligase. The reaction mixtures were transformed into competent HB101 cells, and the transformants were selected for nuclease activity as described above.

Cell fractionation. *E. coli* HB101 harboring plasmids was cultured in Luria broth containing ampicillin (50 μg/ml) to an optical density of 0.4 at 600 nm. The cells were pelleted by centrifugation at 10,000 × *g* and were suspended in an equal volume of ice-cold phosphate-buffered saline, pH 7.2. The cell suspensions were divided into two equal portions, placed into Eppendorf tubes, and centrifuged at 10,000 × *g*. The supernatants were removed by suction.

The cell pellet in the first tube was suspended in 0.1 volume of 0.9% NaCl containing 2,000 U of polymyxin B per ml and then incubated at 4°C for 15 min. After incubation, the cell suspensions were centrifuged for 2 min at 4°C in an Eppendorf centrifuge, and the supernatants obtained were used as the periplasmic cell fraction.

The cell pellet in the second tube was suspended in 0.1 volume of phosphate-buffered saline and sonicated for 5 min at 5-s intervals on ice. The suspension obtained was used as the whole-cell lysate.

Enzyme activity assay. Nuclease activity was assayed by the method of Lachica et al. (9), with a modification using the DNA agar plates (22). To make the DNA agar plates for the assay, DNA agar medium was autoclaved and allowed to cool at 55°C. After the addition of ampicillin, streptomycin, and tetracycline to a final concentration of 50 μg/ml each, 20 ml of the precooled DNA agar medium was poured into petri dishes (20 cm in diameter). Holes (3 mm in diameter) were made in the agar plate, and a 30-μl sample was poured into each hole. The plates were incubated at 37°C for 3 h. The assay scores nuclease activity as a pink zone on a dark blue background. Control experiments using samples obtained from *E. coli* HB101 never showed a positive response in this assay. The nuclease activity of a sample was expressed as the reciprocal of the highest dilution that gave a positive response.

β-Lactamase activity was determined iodometrically at 30°C in 0.1 M phosphate buffer (pH 7.0) by a colorimetric assay as described by Sawai et al. (20). Ampicillin (Sigma Chemical Company, St. Louis, Mo.) was used as a substrate. One unit of β-lactamase activity was defined as the activity capable of hydrolyzing 1.0 μmol of ampicillin per min at 30°C.

ST activity assay. ST activity was assayed in suckling mice as described previously (16). The minimal amount of ST giving a fluid accumulation of more than 0.083 (ratio of intestine weight to body weight) was designated as 1 U, and the enterotoxin titer was expressed as the reciprocal of the highest dilution that gave 1 U of enterotoxin activity. Five mice were used for determination of the ST activity of each sample.

Protein sequence determination. Sequence analyses of STp were carried out in a protein sequencer (model 470A; Applied Biosystems, Foster City, Calif.) connected to a phenylthiohydantoin analyzer. The apparatus was operated according to the instructions of the manufacturer.

RESULTS

The effects of methionine residue substitutions. It was previously presumed but never demonstrated that STp is produced as a 72-amino-acid residue precursor (23). Since extracellular STp is known to be an 18-amino-acid peptide (26), the remainder of the peptide is thought to be cleaved during export to the extracellular medium. However, the STp precursor has not yet been detected. Furthermore, no studies using gene manipulations of the translational initiation site of the STp gene to determine the actual site of translation initiation have been done. Consequently, the existence of the 72-amino-acid residue STp precursor has not yet been proven to exist.

In addition to the amino-terminal methionine residues, there are two additional methionine residues at positions 5 and 53 of the STp precursor as derived from the DNA sequence (Fig. 1). To determine whether the additional methionine residues were used for translation initiation, we substituted these two methionine residues by oligonucleotide-directed site-specific mutagenesis using oligonucleotides 601 and 602, as described in Materials and Methods. Plasmids pKK601 and pKK602 were obtained by mutagenesis using oligonucleotides 601 and 602, respectively. The correct mutations were confirmed by DNA sequence determination (data not shown).

E. coli strains harboring plasmids containing either wild-type or mutant STp genes were cultured in Luria broth at 37°C overnight with shaking. Culture supernatants were obtained by centrifugation, and enterotoxic activities of these supernatants were examined. The enterotoxin titers of the supernatants of *E. coli* harboring Tc-1, pKK601, and pKK602 were 80, 75, and 70 U, respectively. The production of STp from these mutant strains demonstrates that the codons for methionine residues at positions 5 and 53 are not used as translation initiation codons and that STp is synthesized as the 72-amino-acid residue precursor.

Effect of the pro sequence deletion. Many bacterial proteins which are secreted are made as precursors containing an amino-terminal signal sequence or pre sequence. The pre sequence usually consists of fewer than 30 amino acid residues (17) and is cleaved during protein secretion. Since STp is a typical extracellular toxin (5), it is expected to contain a pre sequence. Since the mature STp found in the culture supernatant is composed of 18 amino acids (26), the extended peptide at the amino terminus of STp composed of 54 amino acid residues would be an unusually long pre sequence. Therefore, it is predicted that STp is synthesized in vivo as a precursor which consists of three parts, a pre sequence, mature STp, and a peptide extension between the pre sequence and mature STp termed the pro sequence. However, the exact pre sequence and the role of the pro sequence in the secretion of mature STp remain unresolved. In order to determine the function of the pro sequence and the length of the pre sequence, we deleted the STp gene sequence encoding amino acid residues 22 to 53 from pKK601, as described in Materials and Methods. The obtained plasmid was designated pKK603.

The effect of the pro sequence deletion on STp production was examined by comparing the enterotoxic activities of strains harboring TC-1, pKK601, and pKK603. The cells were grown to an optical density of 0.4 at 600 nm, and the culture supernatants and the cell extracts were prepared as described in Materials and Methods. The culture supernatant and the cell extract of HB101 harboring pKK601 contained ST activities of 20 and 10 U, respectively. These values were almost identical to those of the wild type [*E. coli* HB101(Tc-1)], indicating that pKK601 is a valid wild-type control. The STp deletion mutant activities were detected in the culture supernatant of *E. coli* HB101(pKK603) at approximately 40% that of the wild type. Surprisingly, the STp deletion mutant activity was not detected in the cell extract.

Amino acid sequence determination. In order to determine the amino terminus of the processed STp deletion mutant, the protein was purified as described in Materials and Methods. The elution profile of the purified STp deletion mutant obtained from the Pep column chromatography showed that it was purified to near homogeneity.

Sequential Edman degradation of the purified STp deletion mutant showed that the amino acid residues at positions 1, 2, 3, and 4 from the amino terminus are glutamine, serine, glycine, and serine, respectively. This result demonstrates that the proteolytic processing of the STp deletion mutant occurred between amino acid residues 19 and 20 (Fig. 2).

Localization of the hybrid proteins. To understand the functions of the STp pre and pro sequences, we constructed plasmids pKK604 and pKK605 by fusing the STp gene to the nuclease A gene, as described in Materials and Methods.

Plasmid pKK606 was constructed to provide a cytoplasmic protein control by fusion of the nuclease A gene to the *Bam*HI site of the plasmid pIN-I-A3. Because the *Bam*HI site of pIN-I-A3 is immediately after the translational initiation codon of lipoprotein, the inserted foreign gene product does not possess a signal peptide (12) and therefore should be located in the cytoplasm.

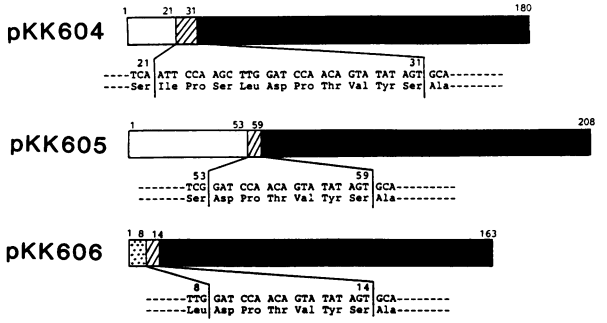

FIG. 3. Structure of the fusion proteins obtained from plasmids pKK604, pKK605, and pKK606. The solid box represents mature nuclease, the open box represents the pre-pro sequence of STp, the stippled box represents the amino-terminal eight amino acid residues derived from a lipoprotein mutant (12), and the hatched box represents the extra sequences generated by the gene manipulations. The nucleotide and amino acid sequences of each extra sequence are shown under the corresponding peptide. The residues are numbered from the amino-terminal residue of each peptide.

The plasmid constructions were confirmed by restriction endonuclease digestion. The hybrid proteins encoded by these plasmids are shown in Fig. 3.

Cells harboring these plasmids were cultured and treated with polymyxin B to obtain the periplasmic fraction. Since the plasmids used in these experiments encode the *bla* gene whose product (β-lactamase) is located in the periplasm, β-lactamase activities of the cultured cells were used as a control to determine the release of periplasmic proteins by the polymyxin B treatment. The results obtained are shown in Table 1. In all the strains examined, the β-lactamase activity (estimated by comparison with whole-cell lysates) was almost completely recovered in the polymyxin B-treated sample. Thus, polymyxin B treatment brought about the release of almost all the periplasmic proteins. Most of the intracellular nuclease activity of the cells harboring plasmids pKK604 and pKK605 was recovered in the periplasmic fractions, but that of cells harboring pKK606 was not recovered in the periplasmic fraction. For comparison, nuclease activities of cell culture supernatants were extremely low in all strains examined.

DISCUSSION

In this paper, we describe the in vivo synthesis and secretion of the *E. coli* heat-stable enterotoxin STp. From the STp nucleotide sequences it has been predicted but never shown that STp is synthesized as a 72-amino-acid residue precursor. There are three methionine residues in the inferred amino acid sequences of the STp precursor at positions 1, 5, and 53 (Fig. 1), suggesting that translation initiation could occur at any of the three residues. We have now demonstrated that the codons for the two methionine residues at positions 5 and 53 are not involved in the initiation of translation. This was shown by substitution of these methionine residues by using oligonucleotide-directed site-specific mutagenesis. We conclude that STp is synthesized as a 72-amino-acid residue precursor.

A recent report indicates that the STh precursor also consists of 72 amino acid residues (19). These results indicate that STp and STh are both synthesized as a pre-pro

TABLE 1. Cellular locations of nuclease and β-lactamase[a]

E. coli strain (plasmid)	Activity of prepn from[b]:					
	Nuclease			β-Lactamase		
	Supernatant	Periplasm	Cell lysate	Supernatant	Periplasm	Cell lysate
HB101(Tc-1)	ND[c]	ND	ND	0.01	1.03	1.28
HB101(pKK604)	ND	16	16	0.01	1.20	1.30
HB101(pKK605)	ND	16	16	ND	1.15	1.35
HB101(pKK606)	ND	4	32	0.01	0.98	1.20

[a] *E. coli* HB101 cells harboring the indicated plasmids were cultured in Luria broth containing ampicillin (50 μg/ml) to an optical density of 0.4 at 600 nm. Culture supernatant was obtained by centrifugation at 10,000 × *g*. The pelleted cells were washed and suspended in an equal volume of phosphate-buffered saline. The suspended cells were equally divided into two tubes. Cells in one tube were treated with polymyxin B to obtain the periplasmic fraction, and cells in the other tube were sonicated to obtain the cell lysate. Nuclease activities and β-lactamase activities of the preparations were assayed by using the methods described in Materials and Methods.

[b] Nuclease activity was expressed as the reciprocal of the highest dilution that gave a positive response in the agar method, and β-lactamase activity was expressed in units per milliliter, as described in Materials and Methods.

[c] ND, Not determined, because undiluted sample showed a negative value.

form, similar to reports of several gram-positive bacterial proteins (7, 10). However, until now, the STp-processing site and the function of the STp pro sequence remained unclear.

The function of the STp pro sequence was studied by deletion mutagenesis; DNA encoding STp amino acid residues 22 to 53 was deleted in pKK603. The amino acid sequence analyses of the STp deletion mutant produced by *E. coli* HB101(pKK603) revealed that the mutant STp precursor is cleaved between amino acid residues 19 and 20. Since the sequence surrounding the cleavage site of the STp deletion mutant is the same as that of native STp (Fig. 1 and 2), cleavage is expected to occur at the same site as in the case of native STp. Judging from the length and amino acid sequence of the cleaved peptide, the enzyme responsible for cleavage is signal peptidase.

Several arguments can be made in support of the prediction that the STp signal sequence is cleaved by signal peptidase. First, as is the case in STp, serine residues have been reported as the amino acid residue just prior to the signal peptidase cleavage site in some bacterially secreted proteins (27). Second, the signal peptidase cleavage site is reported to form a reverse turn (17). Secondary structure analyses of the STp precursor using the predictive rules of Garnier et al. (4) reveal that STp forms a reverse turn around position 19 (data not shown). Third, it was recently found that the STh precursor is also cleaved between positions 19 and 20 by signal peptidase (19).

The role of the ST pro sequence remains to be resolved. ST is secreted efficiently into the extracellular milieu where it is fully active. In contrast, intracellular ST activity is less than 1/100 of extracellular ST activity (5). So, we predicted that the pro sequence functioned in the translocation of STp from the periplasm across the outer membrane, since the translocation from the cytoplasm to the periplasm was brought about by the signal sequence. Surprisingly, deletion of the gene fragment encoding the pro sequence did not abolish extracellular ST activity; in fact, ST activity was approximately 40% of that of the wild type. Therefore, the pro sequence is not absolutely required for the secretion of STp to the extracellular milieu. Consequently, it is doubtful that the pro sequence of STp functions to release mature STp into the extracellular milieu. Interestingly, intracellular ST activity of the mutant could not be detected, indicating that the pro sequence may play a role in protein stability. The results show that the pro sequence is involved in the expression of STp activity. However, the exact role played by the pro sequence remains unknown.

The results obtained from the protein fusion experiments also show the inability of the pro sequence to direct a protein into the extracellular milieu. Hybrid proteins consisting of nuclease A fused to the STp pre-pro sequence (pKK605) were found to be located in the periplasm. Moreover, there was no observable nuclease activity in the culture supernatant. Additionally, the hybrid proteins consisting of nuclease A fused to the STp pre sequence (pKK604) were found to be located in the periplasm (Table 1). Thus, the pre sequence of STp functions as a signal sequence in the transfer of nuclease A from the cytoplasm to the periplasm, and the pro sequence does not bring about secretion of nuclease A from the periplasm to the culture supernatant.

In bacteria, protein synthesis and protein secretion are coupled, and disulfide bonds are formed after the release of the protein into the periplasm (17, 18, 27). The mature STp has three intramolecular disulfide bonds and is stable to inactivation by protease (2, 15). While crossing the inner membrane and just after being released, ST peptides must be very sensitive to proteolysis, because they have not yet properly folded. Recently, reports describing the participation of the pro sequence in an appropriate prototype have been published (28). The possibility remains that the pro sequence of STp may contribute to ST activity by folding the nascent STp polypeptide into a form resistant to cellular proteases. Further studies are in progress in our laboratory to clarify the function of the pro sequence.

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