Two Novel \textit{Streptomyces} Protein Protease Inhibitors

PURIFICATION, ACTIVITY, CLONING, AND EXPRESSION*

(Received for publication, September 5, 1991)

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In contrast to the Gram-negative bacteria, Gram-positive bacteria such as \textit{Streptomyces} lack a mucopolysaccharide cell wall which allows them to produce and secrete a variety of proteins directly into their environment. In an effort to understand and eventually exploit the synthesis and secretion of proteins by \textit{Streptomyces}, we identified and characterized two naturally occurring abundantly produced proteins in culture supernatants of \textit{Streptomyces lividans} and \textit{Streptomyces longisporus}. We purified these 10-kDa proteins and obtained partial amino acid sequence information which was then used to design oligonucleotide probes in order to clone their genes. Analysis of the sequence data indicated that these proteins were related to each other and to several other previously characterized \textit{Streptomyces} protein protease inhibitors. We demonstrate that both proteins are protein protease inhibitors with specificity for trypsin-like enzymes. The presumptive signal peptidase cleavage sites and subsequent aminopeptidase products of each protein are characterized. Finally, we show that the cloned genes contain all of the information necessary to direct synthesis and secretion of the proteins by \textit{Streptomyces} spp. or \textit{Escherichia coli}.

The \textit{Gram-positive} filamentous bacteria of the genus \textit{Streptomyces} are ubiquitous soil microorganisms characterized by a morphologically complex life cycle. These organisms possess the ability to synthesize a variety of secondary metabolites, including many clinically useful antibiotics. In addition, they exhibit exceptional nutritional versatility based on their ability to hydrolyze complex organic compounds in their environment.

The nucleotide sequence(s) reported in this paper have been submitted to the GenBank\textsuperscript{TM}/EMBL Data Bank with accession number(s) M80676 (STI1) and M80677 (STI2).

EXPERIMENTAL PROCEDURES

**Bacterial Strains and Vectors**—The \textit{Streptomyces} species used included \textit{S. lividans} strain 1326, obtained from the John Innes Institute (Norwich, Great Britain), \textit{S. lividans} strain 1326-K12 (18), \textit{S. longisporus} ATCC 23931, and \textit{S. antifibrinolyticus} ATCC 21870. The \textit{Streptomyces} subtilisin inhibitor isolated from \textit{Streptomyces albo-griseolus} (14, 15) and the plasmin inhibitor, plasminostreptin, isolated from \textit{Streptomyces antifibrinolyticus} (16, 17) were used as controls.

The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SSI, \textit{Streptomyces} subtilisin inhibitor; PSN, plasminostreptin; STI1, \textit{Streptomyces} trypsin inhibitor 1; STI2, the gene for STI1; STI2*, TrpB, tryptic inhibitor 2; STI2*, the gene for STI2; API-2c, alkaline protease inhibitor-2c; BPTI, bovine pancreatic trypsin inhibitor; TBST, triis-hydroxymethylamino-methane-buffered saline with Tween-20; RP-HPLC, reverse-phase high performance liquid chromatography; kb, kilobase pair(s); bp, base pair(s).
tomyces vectors employed were pJL350 (19), pJL703 (20), pK21 (21), pSKO2 (18), and pMB157, which has the SCP2* replicon fused to pUC8. S. liuidans 1326 was grown in SL medium (22) supplemented with 1% (v/v) glycerol. S. longiporus was grown in trypticase-soy broth.

The E. coli strains used in this study included TB1 (Bethesda Research Laboratories Focus 6, 1984), and JM101 (23). The E. coli vectors included pUC8 and pUC9 (24), M13 mp10, mp11, mp18, mp19, pUC18, and pUC19 (25), and Charon 30 (26).

**Purification of Extracellular Proteins**—Initially the small protein produced by S. liuidans was designated ST11 (Streptomyces trypsin inhibitor 1). It was identified as an abundant, exported protein in the culture supernatants of S. liuidans grown in SL-glycerol medium for 30 h at 28 °C. Culture supernatants were isolated and filtered, first through cheesecloth and then through a 0.45-μm Nalgel filter. The filtrate was concentrated 10-fold in a hollow-fiber filter apparatus (Amicon, HLP3-20). Solid (NH₄)₂SO₄ was added to 60% of saturation (4°C). The filtrate was concentrated by addition of (NH₄)₂SO₄ to 65% of saturation. The resulting slurry was stirred overnight at 4 °C. Precipitated proteins were recovered by centrifugation (12,000 × g, 20 min), redissolved in 0.01 M sodium phosphate buffer, pH 7.0, and dialyzed against the same buffer. The dialysate was then subjected to electroelution on a 3-μm thick 18% preparative SDS-polyacrylamide gel (27). A guide lane was stained to localize the protein, the corresponding section of the unstained gel was excised, and the protein was electroeluted. This procedure yielded approximately 1 mg of ST11/liter of culture supernatant. The majority of the ST11 sequence data were obtained from the protein isolated in this way.

For functional assays, non-denatured ST11 was isolated from concentrated medium by the addition of (NH₄)₂SO₄ to 65% of saturation. The resulting slurry was stirred for 30 min at 25 °C, and precipitated proteins were recovered by centrifugation (5,000 × g, 30 min). The precipitate was redissolved in 10 mM ammonium acetate buffer, pH 5.0, and dialyzed against the same buffer. The dialysate was applied to an S-Sepharose Fast Flow column (Pharmacia LKB Biotechnology Inc.). 2.5 × 10 cm, equilibrated in 10 mM NH₄Ac, pH 5.0, at 2.5 ml/min. The column was eluted with a linear gradient from 0 to 100 mM NH₄Ac, pH 5.0. ST11 eluted at about 300 mM NH₄Ac. Further purification of the protein could be obtained, when necessary, by reverse-phase high performance liquid chromatography (RP-HPLC).

The small protein observed in culture supernatants of S. longiporus and designated ST12 (Streptomyces trypsin inhibitor 2) was purified by a two-step procedure involving (NH₄)₂SO₄-induced aggregation (65% of saturation) followed by cation exchange chromatography. S. longiporus was grown in tryptase-soy broth medium for 72 h at 28 °C. Under these conditions, the organism produced a large amount of a melanin-like pigment. On addition of (NH₄)₂SO₄, this pigment polymerized to form a dense mat of black fibrous material. Precipitation of ST12 was caught in this material and the entire aggregate was floated. The insoluble material was collected by centrifugation (5,000 × g, 30 min), both from the precipitate and from the mat floating on top of the supernatant solution. The aggregate was re-suspended in 0.055 M tris-buffered saline (pH 7.4) and dialyzed against 10 mM NH₄Ac, pH 5.0. The material was redissolved in 0.01 M sodium phosphate buffer, pH 7.0, and dialyzed against 10 mM NH₄Ac, pH 5.0, and dialyzed against the same buffer. The dialysate was applied to an S-Sepharose Fast Flow column (Pharmacia, LKB Biotechnology Inc.), 2.5 × 15 cm, equilibrated in the dialysis buffer. The column was eluted with a linear gradient from 0 to 250 mM NH₄Ac, pH 6.0. ST12 eluted at about 100 mM NH₄Ac. From 50 to 100 mg of ST12 could be recovered from 1 liter of culture supernatant. Rechromatization of ST12 expressed in S. liuidans, which does not produce melanin, was purified in the same way, except that during the ammonium sulfate fractionation step, the protein precipitated in the usual way.

**Production of Antibodies**—New Zealand White rabbits were inoculated with 100 μg of either gel-purified ST11 or chromatographically-purified ST12. The rabbits were boosted 10 or 14 days later using another 100 μg of the same protein in incomplete Freund's adjuvant. Sera were collected 2 weeks after the boost and tested for reactivity by Western blotting. Anti-ST11 antibodies cross-reacted with ST12 but the equivalent serum was found to block ST12 activity. The antibodies were then blocked with a 500 dilution of the antisera and low amounts of antigen.

**Amino Acid Sequence Analysis**—The Streptomyces proteins were denatured and reduced in a solution of 6 M guanidinium chloride; 500 mM Tris-HCl, pH 8.1, which had been saturated with CH₃CN (≈33% v/v). The samples were then blocked with a 5000 dilution of the antisera and low amounts of antigen. The proteins were recovered by precipitation with ethanol. The precipitated proteins were redissolved in freshly deionized 10 mM urea and then diluted 1:1 with 100 mM NH₄HCO₃. Trypsin (L-1-lysylamide-2-phenyleryl chloromethyl ketone-treated, Cooper Biomedical) was added at 0.2 and 2 h of incubation at 37 °C to yield a final enzyme to substrate ratio of 4:100 (w/w). The peptidases were digested by RP-HPLC on a Brownlee RP300 octyl-silica column (4.6 × 250 mm). The effluent from the column was monitored for absorbance at 214 nm and for fluorescence (excitation at 230 nm, emission above 440 nm). Peptide peaks were collected manually and lyophilized.

The proteins or peptide fragments were subjected to vapor-phase hydrolysis with 5 N constant boiling HCl (Fierce Chemical Co.) for 16-18 h at 110 °C in vacuo. The hydrolysates were analyzed on a Beckman 6300 Amino Acid Analyzer. For sequence analysis, samples were dissolved in either 25% trifluoroacetic acid or 0.1% SDS and subjected to automated Edman degradation in a Beckman model 890 M Sequenator. Released phenylthiohydantoin-derivatives were identified by RP-HPLC on a Beckman Ultrasphere ODS column (4.6 × 250 mm).

**Trypsin Assay**—Trypsin activity was measured using benzoylarginine-p-nitroanilide as substrate. Assays were performed in microtiter plates in a total volume of 200 μl. The buffer was 50 mM Tris-HCl, pH 8.0, 1 mM EDTA and contained 1 mg/ml bovine serum albumin. The enzyme concentration was held constant at 0.2 μM, and the inhibitor and substrate concentrations were varied over the range 0.025-0.5 μM and 0.25-2 mM, respectively. The plate was read at 405 nm in a V max plate reader and the data were collected using an IBM PS/2 model 60. Only assays for which linear rates could be determined were used for subsequent analyses. Aprotinin (Sigma) and bovine pancreatic trypsin inhibitor (BPTI, Sigma) were assayed in the same system for comparison with ST11 and ST12.

**Charon Phage Cloning of the Gene Encoding ST11 (sti)**—A genomic library of S. liuidans 1326 was partially digested with Sau3A, size-fractionated on sucrose gradients, ligated to BamHI-digested Charon 30 DNA, and packaged in vitro using standard techniques (29). The Sau3A fragments used in the cloning experiments were in the size range of 10-25 kb. ST11 was identified as a recombinant phage on the basis that it hybridized with the Charon 30 ST11 sequences by plaque hybridization using a 32P-labeled mixed oligonucleotide probe. The probe was synthesized as a mixture of 24 degenerate sequences complementary to the mRNA sequence predicted from amino acids 92-99 of the mature ST11 protein sequence and taking into consideration the Streptomyces codon usage bias (30). The sequence of the probe was as follows: S'- CTT GAC GCA CTC GTT CGC GAA GGC C3'- T C T C

**Colonial Immunoblot**—Production of ST11 or ST12 by Streptomyces colonies was detected by adsorption of secreted proteins to nitrocellulose filters followed by detection with antibody. Colonies or transformants grown for 3-5 days on RYE agar prior to onset of sporulation were overlayed with nitrocellulose filters (0.2 μm; BA 83, Schleicher and Shuell) and incubated for 4-8 h at 28 °C. The filters were then air-dried, wetted with water, fixed with 10% acetic acid, 25% 2-propanol, and rinsed in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20). The filters were blocked with 0.5% gelatin, washed with TBST, incubated with primary antibody, and then washed with TBST. Antibody binding was detected by reaction with a secondary, biotinylated goat anti-rabbit IgG, and a streptavidin/biotinylated horseradish peroxidase complex (Vectorstain, Vetc-tastain Laboratories, Burlingame, CA). Alternatively antibody binding was detected by binding 35S-labeled protein A (1-5 μg, Amersham Corp., 35 Ci/g) and washed as described above but substituting Western blot wash buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.25% Triton X-100, 0.1% gelatin) for TBST.

**Protein Localization in E. coli**—E. coli strain JM101 containing ST11 plasmid constructions were grown at 37 °C in LB medium containing 100 μg/ml ampicillin at 60% of saturation by using a L500 dilution of the antiserum and low amounts of antigen.

**M. Brawner, personal communication.**
isolating a 1-kb fragment containing the stil gene by partial digestion of plasmid pD1 DNA (see "Results and Discussion") with HindIII, filling in the ends with DNA polymerase I, then partial digestion with NruI. The resulting fragment (−138 to +360) was ligated to Smal/Acl-treated pUC18 to yield pHN1. A PvuII-SalI fragment containing stil was isolated from pHN1, digested with FokI, and the FokI site at +5 was labeled with 32P and then digested with EcoRI to release a 159-bp fragment.

An ST12 probe was constructed by end labeling the BssHII site in the gene encoding ST12 (stil) contained on pSTI2-4262, which consisted of a 1.2-kb SacI fragment from pSTI2-5642 (see "Results and Discussion") inserted in pUC18. The BssHII end-labeled DNA was then released by digestion with EcoRI to release a 421-bp probe.

RESULTS AND DISCUSSION

We examined culture supernatants of Streptomyces spp. for abundantly produced proteins by precipitation with trichloroacetic acid followed by electrophoresis on SDS-polyacrylamide gels and Coomassie Blue staining (Fig. 1). Culture supernatants from both S. lividans and S. longisporus contained 10-kDa polypeptides as major secreted protein species.3

We decided to clone the genes for these polypeptides in order to obtain DNA sequences encoding the proteins, their leader (secretory signal) sequences, and the transcriptional and translational elements responsible for their expression.

Cloning the stil Gene of S. lividans—The 10-kDa protein defined by SDS-PAGE analysis was purified from S. lividans conditioned medium after 30 h in culture by a combination of (NH4)2SO4 precipitation and preparative SDS-PAGE as described under "Experimental Procedures." The reduced denatured protein was digested with trypsin and the fragments isolated by RP-HPLC. Sequence analysis of the resulting peptides provided sufficient information to account for >80% of the protein. The sequence of one of the peptides, Ala-Phe-Ala-Ash-Glu-Cys-Val-Lys, was used in conjunction with a Streptomyces codon usage bias table (30) to design a 24-bp 24-fold degenerate oligonucleotide probe for the mRNA encoding this peptide. This oligonucleotide mixture was used to probe a Charon 30 phage genomic library from S. lividans. Putative clones were detected with a frequency of 3 × 10−4. One recombinant clone, Ch25.5, containing an insert of approximately 18 kb, was selected for further study. A 15-kb BglII-EcoRI fragment of this insert, containing sequences which hybridized with the probe, was subcloned into pUC18 to give the plasmid pD1.

The gene encoding the protein was localized to a 3-kb PstI-BamHI DNA fragment from pD1 and then to a 180 bp Real fragment by probing Southern blots with the mixed oligonucleotide probe. The Real fragment was subcloned into M13 mp10 and sequenced (32). Analysis of this sequence indicated an open reading frame which agreed with tryptic peptide amino acid sequence data. Additional nucleotide sequence was obtained by cloning and sequencing restriction fragments flanking the 180-bp Real fragment. A restriction map for the gene region indicating the sequencing strategy is shown in Fig. 2A. The complete nucleotide and amino acid sequences are shown in Fig. 3A. Amino acid sequence analysis of the full-length protein isolated as described, revealed marked heterogeneity at the NH2 terminus. Sequences were determined consistent with starts at Ser* (−48%, nucleotide +105) and Tyr* (−52%, nucleotide +111). We have assigned Ser as the NH2 terminus of the mature protein since it defines the longest sequence found. The nucleotide sequence also predicts a 30-amino acid stretch that resembles an amino-terminal signal peptide. This sequence contains two potential ATG initiation codons. We have assigned the ATG at position +1 as the initiation codon based on its spacing relative to the putative ribosome binding site AAGGA found 13 bp upstream (underlined, Fig. 3A); however, we cannot rule out the possibility that the ATG at position −9 is used. We designate this protein STI1 and the gene stil.

The 5'-end of the stil transcript was determined by S1 nuclease mapping using a 159-bp EcoRI-FokI DNA fragment (see "Experimental Procedures") and RNA purified from S. lividans grown for 48 h in culture. A unique 5'-end was

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2Two points of interest should be noted with regard to this figure. First, we were not concerned with absolute amounts, i.e. we did not determine whether or not we were quantitatively precipitating every protein in the culture supernatant. Our purpose was merely to try to identify major secreted species. Second, the reader should bear in mind that the sample derived from S. longisporus represents a 72-h time point. Other proteins are evident in the culture supernatant at earlier times; however, these proteins appear to turnover without being replaced, whereas the 10-kDa species continues to accumulate. The net result is that by 72 h, the 10-kDa species accounts for >80% of the total protein in the sample. Other protein bands were evident on the original gel, but were lost during photographic reproduction.

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FIG. 1. SDS-PAGE analysis of Streptomyces culture supernatants. Lane 1 is the trichloroacetic acid-precipitable protein from 0.1 ml of culture supernatant of S. longisporus. Lane 3 is the trichloroacetic acid-precipitable protein from 1 ml of culture supernatant of S. lividans. Lane 2 contains Bethesda Research Laboratories pre-stained molecular mass markers: ovalbumin, 45 kDa; α-chymotrypsin, 25.7 kDa; β-lactoglobulin, 18 kDa; lysozyme, 16 kDa; BPTI, 6.2 kDa; and insulin A and B chains, 2.3 and 3.4 kDa, respectively.

FIG. 2. A, restriction endonuclease map of the S. lividans genomic DNA region surrounding the stil gene. The open reading frame is boxed. Arrows underneath represent the restriction fragments sequenced. B, restriction endonuclease map of the S. longisporus genomic DNA region surrounding the stil gene. The open reading frame is boxed. Arrows underneath represent the restriction fragments sequenced.
A

**Phylogenetic Analysis**

Sequence alignments of the mature NH2-terminal sequences with SSI, PSN, and ST11 were performed using the ClustalW program. The alignment was refined manually to ensure accuracy. The putative signal peptides were identified as those residues immediately preceding the mature protein sequences, with the cleavage site at position +1. The C-terminal sequences were used for the alignment, and the alignment was collapsed at the end to maintain the length of the sequences.

**Nucleotide and Amino Acid Sequences**

The nucleotide and amino acid sequences of ST11 and ST12 are presented in Fig. 3. ST11 has a predicted N\textsubscript{H2} terminus with two conserved cysteine residues, forming a disulfide bond. ST12 has a predicted C-terminal sequence consistent with a signal peptide. The sequences of both ST11 and ST12 are highly conserved, with 66% identity with SSI and 73.4% identity with each other.

**Protein-Protein Inhibitors**

The sequence of ST11 shows similarity to the Streptomyces subtilisin inhibitor (SSI) and plasmin, with a high degree of amino acid identity. ST11 and ST12 exhibit 66% identity with SSI and 73.4% identity with each other and PSN, suggesting that they belong to this class.

**Protein-Protein Inhibitors of PSN**

The alignment of ST11 and ST12 with PSN is shown in Fig. 4. The sequences of ST11 and ST12 are highly conserved, with 66% identity with PSN and 73.4% identity with each other. The alignment was performed using the ClustalW program, and the alignment was collapsed at the end to maintain the length of the sequences.

**Protein-Protein Inhibitors of SSI**

The alignment of ST11 and ST12 with SSI is shown in Fig. 4. The sequences of ST11 and ST12 are highly conserved, with 66% identity with SSI and 73.4% identity with each other. The alignment was performed using the ClustalW program, and the alignment was collapsed at the end to maintain the length of the sequences.

**Protein-Protein Inhibitors of ST11**

The alignment of ST11 and ST12 with ST11 is shown in Fig. 4. The sequences of ST11 and ST12 are highly conserved, with 66% identity with ST11 and 73.4% identity with each other. The alignment was performed using the ClustalW program, and the alignment was collapsed at the end to maintain the length of the sequences.

**Protein-Protein Inhibitors of ST12**

The alignment of ST11 and ST12 with ST12 is shown in Fig. 4. The sequences of ST11 and ST12 are highly conserved, with 66% identity with ST12 and 73.4% identity with each other. The alignment was performed using the ClustalW program, and the alignment was collapsed at the end to maintain the length of the sequences.

**Protein-Protein Inhibitors of SSI and PSN**

The alignment of SSI and PSN is shown in Fig. 4. The sequences of SSI and PSN are highly conserved, with 66% identity with each other. The alignment was performed using the ClustalW program, and the alignment was collapsed at the end to maintain the length of the sequences.

**Protein-Protein Inhibitors of ST11 and ST12**

The alignment of ST11 and ST12 is shown in Fig. 4. The sequences of ST11 and ST12 are highly conserved, with 66% identity with each other. The alignment was performed using the ClustalW program, and the alignment was collapsed at the end to maintain the length of the sequences.

**Protein-Protein Inhibitors of ST11 and SSI**

The alignment of ST11 and SSI is shown in Fig. 4. The sequences of ST11 and SSI are highly conserved, with 66% identity with each other. The alignment was performed using the ClustalW program, and the alignment was collapsed at the end to maintain the length of the sequences.

**Protein-Protein Inhibitors of ST11 and PSN**

The alignment of ST11 and PSN is shown in Fig. 4. The sequences of ST11 and PSN are highly conserved, with 66% identity with each other. The alignment was performed using the ClustalW program, and the alignment was collapsed at the end to maintain the length of the sequences.

**Protein-Protein Inhibitors of ST12 and SSI**

The alignment of ST12 and SSI is shown in Fig. 4. The sequences of ST12 and SSI are highly conserved, with 66% identity with each other. The alignment was performed using the ClustalW program, and the alignment was collapsed at the end to maintain the length of the sequences.

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**Protein-Protein Inhibitors of ST11, SSI, and PSN**

The alignment of ST11, SSI, and PSN is shown in Fig. 4. The sequences of ST11, SSI, and PSN are highly conserved, with 66% identity with each other. The alignment was performed using the ClustalW program, and the alignment was collapsed at the end to maintain the length of the sequences.

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residues between positions 60 and 73, inclusive, only three amino acids (Val<sup>60</sup>, Cys<sup>67</sup>, and Pro<sup>73</sup>) are absolutely conserved among the five proteins. Based on the three-dimensional structures of SSI (37, 38), PSN (39), and STIs 1 and 2,<sup>4</sup> the residues corresponding to 60–73 are part of a loop extending above and away from the packed β-sheet structure of the rest of the molecule. The loop is restrained by a disulfide bond between Cys<sup>67</sup> and Cys<sup>74</sup> and terminates with Pro<sup>73</sup> which starts β-strand 4. In the SSI-subtilisin complex, this loop contains most of the residues in contact with the proteolytic enzyme (40). In addition to these structural data, mutation of the P<sub>1</sub> residue has in determining specificity.

The hypervariability seen in the enzyme contact region observed with these inhibitors is similar to that observed by Laskowski and colleagues (42,43) in their exhaustive study of the ovomucoid third domain. As with ovomucoid, residues contributing to the basic structure of the inhibitors are highly conserved (e.g. the 4 Cys, Pro<sup>73</sup>-Val<sup>74</sup>, etc.), whereas those that influence the function of the molecule vary widely. In the Streptomyces inhibitors, it seems likely that the sequence variation within this region results from evolutionary “tailoring” of the inhibitors for the best fit with their cognate proteases. We do not know the natural protease targets of STI1 and STI2; however, as in PSN, these proteins have a basic amino acid (Arg in STI1, Lys in STI2) in the P<sub>1</sub> position. Thus, we would expect them to be inhibitors of trypsin-like proteases. To test this, we compared the ability of STI1, STI2, BPTI, aprotinin, and PSN to inhibit trypsin (Fig. 5). Clearly, both STI1 and STI2 are capable of inhibiting trypsin. They can also inhibit plasmin (data not shown).

Expression of stil1 and stil2—During the course of characterizing recombinant STI2 isolated from 72-h cultures of <i>S. lividans</i>, we performed NH<sub>2</sub>-terminal sequence analysis on the purified protein. We found that approximately 77% of the recombinant protein started with the Ser at position 2 (nucleotide +106) rather than the Ala at position 1 (nucleotide +102, Fig. 3B), as previously defined for the native protein isolated from <i>S. longisporus</i>. The predominance of Ser<sup>2</sup> at the NH<sub>2</sub> terminus of STI2 produced by <i>S. lividans</i> is consistent with the Ser NH<sub>2</sub> terminus which we identified for the <i>S. lividans</i> homolog STI1 (see Fig. 3A). An additional 18% of the STI2 produced by <i>S. lividans</i> began with Leu<sup>4</sup> (nucleotide +109) analogous to the native protein. Surprisingly, a small but reproducible proportion (~5% of the protein) had three additional amino acids (Ala-Ala-Pro-Ala<sup>1</sup>-Ser<sup>2</sup>-) at the NH<sub>2</sub> terminus. This result suggested that the NH<sub>2</sub> termini defined by the proteins we isolated after extended culture (72 h) might be the result of extracellular degradation by aminopeptidases subsequent to the initial signal peptide cleavage event upstream of the putative +1 position. To examine this possibility, we analyzed samples of <i>S. longisporus</i> conditioned medium after various times in culture by SDS-PAGE. At early times (24 h), we observed a major species of STI2 migrating with an apparent molecular weight ~1,000 higher than that observed for STI2 at later times. After 48 h, this 11-kDa species and the normal 10-kDa species were present in approximately equal amounts. By 72 h, only the smaller 10-kDa species was present (data not shown). We isolated the higher molecular mass species from a 24-h culture and determined its NH<sub>2</sub> terminus. This protein had an additional 6 residues corresponding to 60–73 is a loop extending above and away from the packed β-sheet structure of the rest of the molecule. The loop is restrained by a disulfide bond between Cys<sup>67</sup> and Cys<sup>74</sup> and terminates with Pro<sup>73</sup> which starts β-strand 4. In the SSI-subtilisin complex, this loop contains most of the residues in contact with the proteolytic enzyme (40). In addition to these structural data, mutation of the P<sub>1</sub> residue has in determining specificity.

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* M. Lewis, personal communication.
tures. Since the ST11 and ST12 produced by E. coli appeared to have the same size as the mature products from Strepto-
myces, it seemed likely that similar post-translational pro-
cessing events were occurring. Moreover, the proteins were localized to the periplasmic space of E. coli. These results indicate that the Streptomyces translational elements can be utilized by E. coli and that Streptomyces-derived secretion information functions in E. coli.

Acknowledgments—We wish to thank G. Sathe and J. Sutton for the synthesis of oligonucleotides, M. Brawner and M. Lewis for communication of unpublished results, and M. Brawner, M. Blackburn, and T. Meek for helpful discussions and critical reading of this manuscript.

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