Structure and Expression of a Gene Encoding the Precursor of Subtilin, a Small Protein Antibiotic*

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A gene has been cloned from Bacillus subtilis ATCC 6633 that encodes a 56-residue peptide precursor for the 32-residue peptide antibiotic, subtilin. The precursor contains serines, threonines, and cysteines at positions that permit them to undergo a series of dehydration and cross-linking steps to give the mature antibiotic peptide which contains the unusual amino acids lanthionine, β-methylanthionine, D-alanine, dehydroalanine, and dehydrobutyryl. The precursor peptide contains a leader region that has an unusual hydrophilic character for an exported protein. The subtilin gene is expressed from a monocistronic transcriptional unit as identified by S1 mapping of the gene transcript. The (~35) region of the promoter is not typical of Bacillus subtilis vegetatively-expressed genes. The subtilin transcript was present in very low amounts during exponential growth, but in large amounts during stationary phase; at which time mature subtilin peptide and antibiotic activity were also observed. The subtilin transcript contains a cleavage-sensitive region that overlaps the ribosome binding site. The primary transcript has an unusually long half-life of about 45 min. These observations confirm that subtilin is derived from a small protein that is synthesized on ribosomes. Small biologically active peptides play many important and useful roles in biological systems. Inasmuch as the sequence structures of peptides, and hence their functions, are easily altered by genetic engineering and related techniques, it is appropriate to expect that it will eventually be possible to design and construct analogs of natural peptides that have improved, modified, or novel functions.

A barrier to achieving this is the fact that many such peptides are assembled by nonribosomal processes and contain unusual amino acids that are not among those which are defined by the genetic code. Moreover, these unusual amino acids often play key roles in the biological activity of the peptides in which they are found. Construction of analogs of such peptides would apparently require difficult synthetic organic procedures. An important exception exists for peptides that are made ribosomally and then converted into unusual forms by post-translational processes. When one considers the fact that post-translational processing of peptides has the effect of side-stepping the limitations imposed by the genetic code, allowing the inclusion of novel amino acids into otherwise mundane peptides, it becomes important to determine whether they are dedicated processes, or whether they could be directed toward other peptides in a controlled way. An understanding of how to do this could greatly broaden the repertoire of amino acids that the genetic engineer could aspire to incorporate into his designs.

With these considerations in mind, we have been studying the natural peptide antibiotics, nisin and subtilin, which contain 34 and 32 residues, respectively. Despite an extraordinary proportion of unusual amino acids (over one in three, including D-alanine, lanthionine, β-methylanthionine, dehydroalanine, and dehydrobutyryl), the observations that nisin appearance can be blocked by protein biosynthesis inhibitors (1), and that subtilin precursor peptides can be detected with antibodies against mature subtilin (2), suggest that nisin and subtilin are synthesized by a ribosomal mechanism, followed by post-translational modifications. It has been suggested that these modifications may include dehydrations of serines and threonines to double-bond dehydro forms, some of which could undergo electrophilic addition involving stereoinversion to neighboring cysteine residues to generate thioether cross-linkages and D-amino acids (3). The primary structures of mature nisin and subtilin, which contain five different unusual amino acids, are shown in Fig. 1. If the proposed biosynthetic mechanism is correct, it means that the post-translational system available for these peptides alone has expanded the amino acid repertoire beyond that of the genetic code by 25%. These novel amino acids seem to be functionally important, inasmuch as these peptides can be autoclaved at pH 2 without activity loss (4), and their mechanism of bacteriostatic action may involve electrophilic addition of dehydro residues to sensitive sulfhydryl groups in bacterial membranes (5). It seems likely that this remarkable heat stability is a consequence of the thioether cross-linkages. Moreover, the availability of an electrophilic group, which is notably absent among the ordinary amino acids, provides new scope for the kinds of chemical properties that could be engineered into peptides. Testimony that the reactivity of these electrophiles is highly constrained and specifically targeted is given by the fact that nisin is used as a food preservative throughout the world (6) and is present in many dairy products because nisin-producing Streptococcus lactis is used in dairy fermentations (4). The mechanism of nisin bacteriostatic action appears to be similar to that of nitrite (7, 8) which is another important food preservative. Analogs of nisin and subtilin may prove to be functionally superior to the natural forms, but their structural complexity has prevented their synthesis (9).

In this paper, we report the cloning of a gene from Bacillus subtilis ATCC 6633 strain that encodes exactly the precursor

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J053767.† Will submit this paper as part of a Ph.D. thesis at the University of Maryland.

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9508
sequence required to generate mature subtilin by the scheme of dehydrations and cross-linkages described above. We have studied the structure and expression of this gene and found the sequence consistent with the role of directing the synthesis of the subtilin precursor peptide. The significance of these results is considered.

MATERIALS AND METHODS

Organism and Culture Conditions—B. subtilis ATCC 6633, a subtilin-producing strain, was obtained from the American Type Culture Collection, Rockville, MD. It was cultured in the high sucrose Medium A of Nishio et al. (2), originally described by Feeney et al. (10). It contains (per liter) 100 g of sucrose, 11.7 g of citric acid, 4 g of NaSO₄, 4.2 g of (NH₄)₂HPO₄, 5 g of yeast extract (Difco), 100 ml of a salt mixture (7.82 g of KCl, 4.18 g of MgCl₂·6H₂O, 0.645 g of MnCl₂·4H₂O, 0.49 g of FeCl₃·6H₂O, and 0.208 g of ZnCl₂ in 1000 ml of H₂O), and sufficient NH₄OH to bring the pH to 6.8-6.9 per liter. Stocks were maintained on LB plates (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl per liter) containing 1.5% agar.

Clone Isolation and Hybridization Procedures—A subtilin gene probe was designed based on the putative amino acid precursor sequence of subtilin. The subtilin molecule contains only 32 amino acids and does not contain any regions of low codon degeneracy. Therefore, instead of preparing a probe mixture which contained all possible sequences encoding a short stretch of amino acids in the subtilin precursor, a single long probe was synthesized according to the strategy of Lathe (11). Ambiguous positions within codons were chosen by educated guess, according to a codon frequency usage table constructed from the known B. subtilis gene that codes for α-amylase (12). Because one cannot predict the sequence homology between the probe and the target gene sequence, hybridization and wash conditions must be optimized empirically. The 96-mer "guessmer" was end-labeled using polynucleotide kinase, purified on disulfide cross-linked polyacrylamide gels, and hybridized to total mRNA at several different temperatures, and unhybridized single-strand nucleic acid was degraded using nucleases S1. The product was separated by electrophoresis on a denaturing sequence gel alongside a set of dideoxy sequence analysis reactions generated using the same synthetic oligonucleotide as primer. The location of the protected labeled DNA fragment with respect to the sequence analysis lanes identified the end of the mRNA.

RESULTS

Characterization of the Subtilin Gene Probe and Its Use for Isolation of Clones—A synthetic 96-nucleotide subtilin gene probe guessmer was hybridized to genomic ATCC 6633 DNA that had been restricted with EcoRI, examined by electrophoresis, and blotted. A variety of stringencies were used in both the hybridization and wash steps. The cleanest signals were obtained using hybridization conditions of 52 °C in 6× SSC and wash conditions of 50 °C in 2× SSC. Under these conditions, one strong band was observed at 7.6 kb, along with several weaker bands. These same hybridization and wash conditions were used to screen an unamplified genomic library of ATCC 6633 constructed in bacteriophage Aγ1. Approximately 80 clones that hybridized strongly to the probe were selected. Such clones appeared with frequencies of 1 per 100 and 1 per 1000, which is about 3-fold higher than would be expected for a single-copy gene without any cloning artifacts. DNA was isolated from about 2 dozen of these, and all of the restriction digests were hybridized to the same probe under the same conditions. A majority of the clones contained a 7.6-kb EcoRI restriction fragment. One of these was subcloned into pUC9, to give a 7.6-kb EcoRI insert. This insert was purified, labeled, and hybridized back to the genomic digest under stringent conditions. A single band of 7.6 kb was observed.

Restriction and Sequence Analysis of the Region Which Hybridizes to the 96-Mer Subtilin Gene Probe—The 7.6-kb EcoRI insert was subjected to mapping with restriction enzymes, then cut with XbaI, and the EcoRI-XbaI fragment was subcloned into the phagemid pTZ 19U. This fragment was mapped further. The gels from the restriction digests were blotted and hybridized with the 96-mer probe to identify fragments which contained the hybridizing sequence. These were cloned into M13 and sequenced. The results of restriction mapping and sequencing strategy are shown in Fig. 2. The sequence was searched for homology to the subtilin gene probe and also computer-translated in all reading frames. These were searched for the putative subtilin precursor sequence. A perfect match was found, which contained the exact sequence of 32 residues. The sequence encompassing this region is shown in Fig. 3.

Characterization of the RNA Transcript from the Subtilin

The abbreviations used are: kb, kilobase pairs; bp, base pairs.
Sequence—We next proceeded to establish whether this sequence is part of an expressed gene, and, if so, to characterize the transcript. The availability of the sequence of the putative structural gene permitted us to synthesize perfectly homologous probes, which could be used to search for mRNA transcripts, and to aid in S1 mapping studies. For these purposes, we used the synthetic 20-mer oligonucleotides that we had previously found to be effective in S1 mapping studies. For these purposes, the (-10) region of the promoter was identified and the (-35) region was also seen, which could be a result of degradation or processing of the primary transcript. The terminal nucleotide corresponding to (-10) region of the promoter was identified and is also indicated in Fig. 3, and is typical of vegetative prokaryotic promoters. However, the corresponding (-35) region is not typical, suggesting that this gene may be subject to complex regulation or may be expressed at times other than vegetative growth. The identity of the 3’ end of the transcript can also be derived from the data shown in Fig. 4. It appears that termination is not completely specific, since several bands are present. Fig. 4 shows the terminator sequence identified by this result. It is a typical p-independent prokaryotic terminator. The presence of appropriate promoter, initiator, and terminator sequences indicates that this is a transcriptional unit, and that the RNA revealed by the S1 mapping experiments is the actual primary transcript. Whereas this shows that most of the subtilin mRNA is probably transcribed from this transcriptional unit, it does not rule out the possibility that some is transcribed as part of a longer polycistronic message, especially from read-through from an upstream region, since an upstream terminator has not yet been identified.

The Subtilin mRNA Has a Site Which Is Sensitive to Cleavage—The autoradiogram in Fig. 4 shows a faint band of smaller size than the complete transcript; this band might be due to nonspecific hybridization, or it may indicate that the mRNA had been partially degraded during isolation, or that the mRNA contains a specific processing site. The S1 mapping experiments in Fig. 4 show a cluster of S1 cleavage sites

**Fig. 2. Restriction map and sequencing strategy of region containing subtilin gene.** A 7.6-kb EcoRI fragment obtained from a λ clone isolated from a genomic library constructed from *B. subtilis* ATCC 6633 was subcloned into pUC9. An EcoRI-XbaI fragment of this was subcloned into pTZ 19U and M13 (mp18 and 19). The nucleotide sequence was partially determined by the dideoxy method according to the strategy shown. Synthetic oligonucleotides were used in some cases as indicated. The sequence obtained is shown in Fig. 3.

**Fig. 3. Sequence of a portion of 7.6-kb EcoRI fragment that contains the gene encoding the subtilin precursor peptide.** The portion which corresponds to the leader region that does not appear in the mature peptide is overscored by *asterisks*. The sequence of the mature region is numbered from *t* in 3’ to *t* in 5’. The sequence was determined by S1 mapping data shown in Fig. 4. The (-10) region corresponds closely to a consensus prokaryotic promoter (TATAAT) as observed in other bacteria (25). A typical prokaryotic consensus sequence (−35) is (TTGACA), which is quite different from that shown here. The putative ribosome binding site is labeled as r.b.s. and encompasses a 12-bp sequence that is typical of those observed in *B. subtilis* (26); it is positioned appropriately so that translation initiation would begin at the immediately downstream Met codon. The cleavage-sensitive sites are denoted by the letter s below the r.b.s. The location of the terminator sequence was determined by S1 mapping data shown in Fig. 4. The (-10) region corresponds closely to a consensus prokaryotic promoter (TATAAT) as observed in other bacter (25).
in the region of the putative ribosome binding site that is discussed below. These cleavage products were present in RNA that was S1-mapped immediately after isolation, but their proportion increased significantly during 1 week of storage as a precipitate in ethanol at −20 °C. The presence of the cleavage products in freshly prepared RNA suggests that cleavage is produced by an in vivo process, but its increase during storage suggests that the sensitive region is also subject to nonenzymatic cleavage.

The Gene Sequence Predicts That Subtilin Is Synthesized as a Peptide Precursor That Undergoes Post-translational Cleavage—The sequence of this region which encodes the mature subtilin sequence is typical of known B. subtilis ribosome binding sites. There is a terminator codon immediately following the lysine residue of the mature subtilin sequence. Therefore, the subtilin precursor peptide has a leader region, but no tail. This leader region undoubtedly plays a role in transport of subtilin outside the cell, but it is unusual in comparison to leader sequences of other prokaryotic exported proteins as is discussed below.

The Subtilin Gene Is Expressed Mainly during Late Growth Stages—It has been unclear whether the appearance of mature active subtilin only during very late stages of cell growth is due to the fact that expression of the genes involved with subtilin production is delayed until then, or whether it is due to a lag between the time that the precursor is synthesized and the time that processing and transport of the mature peptide are complete. The availability of hybridization probes which are specific for the subtilin mRNA allows this question to be addressed. A growth curve for ATCC 6633 cells growing in Medium A is shown in Fig. 5. The cells grow to a high density, reflecting the high levels of sucrose added to induce subtilin production. The supernatant fluid from the growth medium was tested for subtilin activity at different times during growth, and the presence of actual subtilin peptide was detected by electrophoresis and staining. These results are shown in Fig. 6.

To determine the corresponding pattern of subtilin mRNA synthesis, total RNA was isolated from cells isolated at different times during growth, then separated by electrophoresis, and stained. The stained gel showing the RNA populations at each time is shown in Fig. 6. A sample of RNA isolated from vegetative cells of B. subtilis strain 168 is included. The RNA preparations look substantially undegraded, in that the lanes show little background. The sample at 22 h has a bizarre appearance, suggesting the presence of substances that interfere with the electrophoresis. This was consistently obtained in every preparation of RNA isolated at this growth stage. The culture supernatant at this time was thick and viscous, possibly as a consequence of sucrose conversion to polymers. This was a transient phenomenon. The RNA gel was subjected to Northern blot analysis with the synthetic 20-mer as a probe. The result is shown in Fig. 6. It shows that there is a strong correlation between the appearance of subtilin peptide, subtilin activity, and subtilin mRNA.
were grown under the same conditions as described in Fig. A shows total protein in the supernatant fluids as revealed by silver staining. The lane labeled standard (std) consisted of a mixture of proteins of known molecular weight. A band corresponding to the molecular weight of subtilin was present at 22 and 32 h, but was absent at 12 h. Subtilin antibiotic activity correlated with the appearance of this band; activity was indicated by (+). B shows the total RNA populations in the cells as revealed by ethidium bromide staining. The gel in B was electrophoresed and hybridized to the 5' end-labeled 20-mer probe that was used for S1 mapping in Fig. 4; the autoradiogram is in C. A strongly hybridizing band appeared in the 22-h sample. A weaker band was present in the 32-h sample. Total RNA isolated from vegetative cells of B. subtilis 168 did not give a signal. D shows a plot of RNA preparations from 8, 10, and 12 h that were treated similarly, except they were hybridized with a 1.1-kb DNA fragment that had been nick-translated to high specific activity. The smaller band was of the same size as the band in C. The identities of these are discussed in the text.

None is present at 12 h of growth, whereas all are present at 22 h. Reference to the growth curve shows that the cells are no longer in true exponential phase after 12 h, so one can conclude that the subtilin gene is not expressed significantly under conditions of low population pressure. Conversely, one can state that there is a great deal of subtilin gene expression during early stationary phase, and expression continues to be significant for a long time. It is tempting to suggest that the induction of subtilin gene expression is correlated with the phenomenon of sporulation, since sporulation is a process that is not initiated until stationary phase. However, microscopic examination of ATCC 6633 after 32 h of growth in Medium A showed no evidence of the sporulation process having been initiated. This organism is nevertheless capable of sporulation, as was established by streaking cells onto potato agar plates and incubating at 37 °C for several days, giving high levels of sporulation. The absence of sporulation in Medium A is probably a consequence of its high carbohydrate content, which commonly suppresses sporulation in bacteria (18).

The results in Fig. 6 show that significant induction of subtilin mRNA has occurred within 22 h of growth, but the absence of a hybridization signal at earlier growth times does not permit an estimate of the relative level of induction, nor does it provide any information about whether the gene is expressed at all during earlier times. This was examined with a more sensitive probe prepared by nick translation of a 1.1-kb DNA fragment that contained the entire subtilin gene. This was hybridized to Northern blots of RNA isolated after 8, 10, and 12 h (Fig. 6). The autoradiogram shows a band at the position of subtilin mRNA, of about equal intensity, at all three times. It also shows another band of larger size. Another gel was prepared that was highly overloaded with the 12-h RNA, which was then hybridized with the subtilin gene-specific 20-mer probe. After a sufficiently long exposure to reveal a signal, only the lower band could be seen (data not shown). It appears that the 1.1-kb probe contains sequences that correspond to another expressed gene in addition to the subtilin gene. These results show that the subtilin gene is expressed at a low level during exponential growth. Comparison of hybridization intensities among several experiments indicated that the level of induction in stationary phase compared to exponential phase is of the order of 100-200-fold (comparisons not shown).

The Subtilin mRNA Is a Long Lived Species—The lifetime of the subtilin mRNA was examined by adding rifampicin to a cell culture at a selected growth stage to inhibit RNA synthesis, removing samples at intervals, isolating total RNA, electrophoresing on polyacrylamide gels, and doing Northern analysis with the 20-mer subtilin gene probe to detect the presence of subtilin mRNA that remained after rifampicin addition. This experiment was done using RNA isolated from cells grown for 12 and 22 h, respectively (cf. growth curve in Fig. 5). Fig. 7 shows that the level of mRNA remained relatively constant for at least 30 min after rifampicin addition and was not detectable after 60 min, suggesting the mRNA half-life is of the order of 45 min. Although the amount of subtilin mRNA (Fig. 6) is much higher at the 22-h time, which is stationary phase, the difference does not seem to be accountable by a difference in mRNA half-life. Moreover, it appears that at both growth stages, mRNA molecules persist without substantial change in mRNA half-life. Moreover, it appears that at both growth stages, mRNA molecules persist without substantial change for at least 30 min and are then degraded over a relatively short time, implying a slow or delayed modification followed by rapid degradation of the modified form.

The Hydropathic Nature of the Leader Region of the Subtilin Precursor Peptide Is Mainly Hydrophilic, Whereas the Structural Region Is Hydrophobic—The hydropathic profile of the subtilin precursor peptide was calculated as shown in Fig. 8.

Fig. 6. Northern analysis of RNA populations isolated at different stages of growth of B. subtilis ATCC 6633. Cells was grown under the same conditions as described in Fig. 5. At indicated times, culture samples were removed and centrifuged. Total RNA was isolated from the cell pellet, and the supernatant fluid was used as a source of extracellular proteins, which were analyzed as described under "Experimental Procedures."
There is a striking difference between the leader region and the structural region. When the sequence is considered 1 residue at a time, the leader region alternates between strongly hydrophobic and strongly hydrophilic residues; but when the profile is smoothed by taking an average over several residues, the N-terminal end is neutral and becomes quite hydrophilic near the beginning of the structural region. Conversely, the structural region is strongly hydrophobic, especially when one considers that several of the hydrophilic precursor residues such as Ser, Thr, and Cys are converted to comparatively hydrophobic lanthionine and β-methyllanthionine residues. The leader region of exported prokaryotic proteins usually contains a distinctly hydrophobic region and basic residues (19). Here, the leader region is primarily neutral to hydrophilic, with no clustering of hydrophobic residues, and a majority of the charged residues are acidic.

**DISCUSSION**

The results presented here answer many long standing questions about the way that cells synthesize antibiotic peptides such as subtilin and nisin. The speculation, supported by some earlier evidence, that subtilin is synthesized on ribosomes as a precursor that undergoes extensive post-translational modification (cleavage, dehydrations, and formation of thioether cross-linkages) is confirmed here by isolation of a gene that encodes the exact sequence of the appropriate precursor peptide. Acidic residues are marked (−) and lysine residues (+). A hydropathic index of 4.5 (horizontal dotted lines) is neutral. Indices connected by solid lines are for unmodified precursor residues. An attempt was made to estimate the hydropathic character of residues in the post-translationally modified peptide by substituting Ala for Ser and Thr, and Met for Cys in the sequence calculated by the algorithm (indices connected by dotted lines in B). The N-terminal tryptophan of the mature subtilin peptide is indicated at residue position 25.

![Hydropathic profile of subtilin precursor peptide](image)

**FIG. 8.** Hydropathic profile of subtilin precursor peptide. Hydropathic profiles were calculated using the algorithm of Kyte and Doolittle (28). A shows the hydropathic index of each individual residue of the subtilin precursor peptide. Acidic residues are marked (−) and lysine residues (+). B shows the index of the central residue of an 11-mer moving segment, calculated as an average of the segment. A hydropathic index of 4.5 (horizontal dotted lines) is neutral. Indices connected by solid lines are for unmodified precursor residues. An attempt was made to estimate the hydropathic character of residues in the post-translationally modified peptide by substituting Ala for Ser and Thr, and Met for Cys in the sequence calculated by the algorithm (indices connected by dotted lines in B). The N-terminal tryptophan of the mature subtilin peptide is indicated at residue position 25.
pending on the precise details of the processing mechanism, it may be possible to insert appropriate precursor residues at preselected sites that are destined to be dehydrated, merely by placing them behind an appropriate leader peptide sequence. An argument against this is the occurrence in nisin of an unmodified serine residue at position 29, suggesting there is not a totally blind dehydration of every serine and threonine in the precursor sequence, although other interpretations are possible. The answers to these questions will ultimately determine the practicality of developing a routine approach to directed introduction of novel amino acids into biosynthesized peptides.

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