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NOTES

Translation and Processing of *Bacillus amyloliquefaciens* Extracellular RNase

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***Bacillus amyloliquefaciens* extracellular RNase has been previously cloned and expressed in *Bacillus subtilis*. Site-specific mutagenesis experiments have identified codon −39 as the start site of translation. We have determined the primary signal peptide cleavage site of preprobarnase and propose a pathway for the conversion of probarnase to mature barnase.**

Bacillus amyloliquefaciens secretes an RNase (barnase), the mature form of which is a monomer of M_r 12,382 consisting of 110 amino acids (4). *B. amyloliquefaciens* also accumulates a low level of a probarnase in the growth medium; this probarnase contains four extra amino acids at the amino terminus (7). In addition, a specific inhibitor of barnase, called barstar, is produced in *B. amyloliquefaciens* (6). The gene coding for barnase was cloned following Tn*917* insertional mutagenesis (7), and inactive barnase was expressed in *Escherichia coli* and *Bacillus subtilis*. Attempts to express active barnase in both organisms were unsuccessful, possibly because the RNase activity of the enzyme was lethal (8). However, active barnase has been recently expressed by coexpressing it with barstar (the specific inhibitor of barnase from *B. amyloliquefaciens* [3]). We are interested in using the barnase signal sequence for the secretion of heterologous proteins from *B. subtilis*. As a first step towards this goal, we report here on the translation and processing of barnase in *B. subtilis*.

The amino acid sequence deduced from the DNA sequence of the barnase gene shows an amino-terminal signal peptide coding region followed by a short polypeptide pre-

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CGC TAG ATA AAA AGT ACT ATT TTT AAA TTC TTT CTA TTC CTT TCT TTC

                                                        TAG
                                                         ↑
GTT GCT GAT ACA ATG AAA AGG AAT CAG CTT CAC ATG ATG AAA ATG GGA

            GCG      TAA
             ↑        ↑
GGT ATT GCT TTG AAA AAA CGA TTA TCG TGG ATT TCC GTT TGT TTA CTG
          fMet-Lys-Lys-Arg-Leu-Ser-Trp-Ile-Ser-Val-Lys-Leu-Leu
       -40                                              -30

GTG CTT GTC TCC GCG GCG GGG ATG CTG TTT TCA ACA GCT|GCC AAA ACG
Val-Leu-Val-Ser-Ala-Ala-Gly-Met-Leu-Phe-Ser-Thr-Ala|Ala-Lys-Thr
                        -20                         |PRO I

GAA ACA TCT TCT CAC AAG|GCA CAC ACA GAA|GCA CAG GTT ATC AAC ACG
Glu-Thr-Ser-Ser-His-Lys|Ala-His-Thr-Glu|Ala-Gln-Val-Ile-Asn-Thr
-10                    |PRO II         |MAT
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FIG. 1. DNA sequence and deduced amino acid sequence of the translation initiation region and signal sequence of barnase (7). PRO I, PRO II, and MAT indicate the N termini of pro I barnase, pro II barnase, and mature barnase, respectively (see text). Codons are numbered relative to the mature barnase N terminus. Mutations introduced at codons −46, −39, and −37 are shown above these codons. Experiments described in the text suggest that the translational initiation site occurs at −39 (TTG). The ribosome-binding site is underlined. fMet, Formylmethionyl.

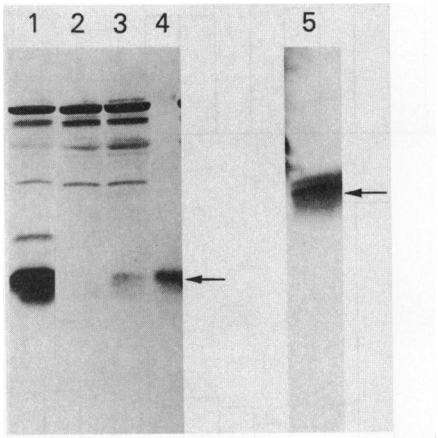

FIG. 2. Immunoblot showing expression of inactive barnase in *B. subtilis* following mutagenesis at codons −46, −39, and −37. The barnase gene was carried by the shuttle vector pUC9/pC194 in all cases. Lane 1, Production of inactive barnase prior to mutagenesis; lane 2, mutation −37; lane 3, mutation −39; lane 4, pro II barnase standard (arrow); lane 5, mutation −46, from a different gel than that of lanes 1 through 4. The arrow beside lane 5 indicates mature inactive barnase. Higher-molecular-weight bands are due to cross-reacting proteins.

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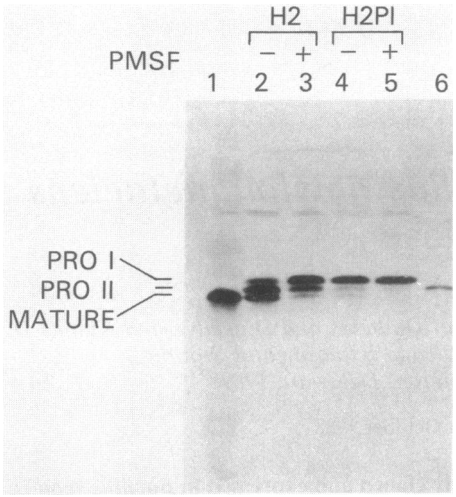

FIG. 3. Immunoblot showing pro I, pro II, and mature barnases isolated from *B. amyloliquefaciens* H2 and H2P1 culture supernatants following growth at 20°C. H2P1 is a secreted protease-deficient mutant (5). Lane 1, Mature barnase standard; lanes 2 and 3, culture extracts from H2 grown in the absence or presence of 1 mM PMSF; lanes 4 and 5, culture extracts from H2P1 grown in the absence or presence of 1 mM PMSF; lane 6, pro II barnase standard.

ceding the mature protein (Fig. 1). DNA sequence analysis has revealed four putative start sites of translation at codons −47, −46, −44, and −39 preceding the mature protein. However, only codon −39 is preceded by potential ribosome-binding site AAATGGGAGGT (binding energy, −15.2 kcal/mol [~−64 × 10^3 J/mol]), which resembles a bacillus translation initiation site (2). To determine which codon is used for translation initiation, codons −46 and −37 were mutated to stop codons and codon −39 was mutated from TTG to GCG. Oligonucleotide-directed mutagenesis (9) used as template a 1.3-kilobase *Eco*RI fragment containing the inactivated barnase gene (His-102 to Gln) in M13mp11 (8). The sequences of the oligonucleotides used to produce mutations were (specific base changes are underlined) CTTC ACATGTAGAAAATGGG (codon −46), AGGTATTGCT GCGAAAAAACGA (codon −39), and GCTTTGAAATAA CGATTA (codon −37). The mutant barnase genes were sequenced (10), subcloned into the shuttle vector pUC9/pC194 in *E. coli*, and subsequently transferred to *B. subtilis*. Immunoblots (1) were stained with antibarnase serum; the production of barnase antigen in *B. subtilis* is shown in Fig. 2. Expression was unaffected by a stop codon at −46, but it was abolished by a stop codon at −37 and drastically reduced by alteration of codon −39 from TTG to GCG. Therefore, translation must initiate predominantly from codon −39 in *B. subtilis*. There was low-level expression following mutagenesis at codon −39; whether this was due to initiation upstream of this point or to a low level of initiation at −39 is not clear. We consider the possibility of initiation at the ATG codon at −44 unlikely for lack of a ribosome-binding site.

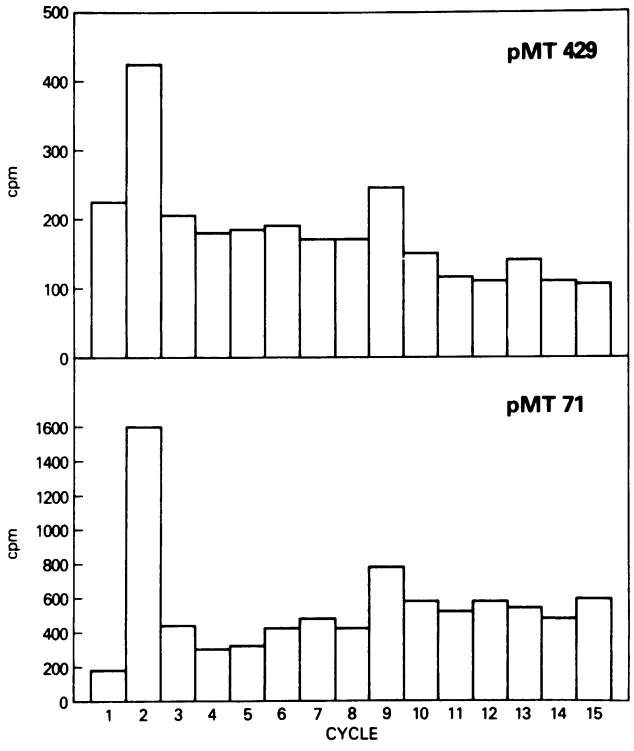

FIG. 4. Determination of the N-terminal sequence of pro I barnase by automated Edman degradation. Pro I barnase was isolated from culture supernatant of *B. subtilis* carrying the indicated plasmids after growth of the culture in [^{3}H]lysine. The graphs show radioactivity released after each degradation cycle.

When *B. amyloliquefaciens* is grown at 30°C to high cell density, mature barnase is the predominant product. A minor product is a probarnase containing four extra amino acids at the amino terminus (Ala-His-Thr-Glu), henceforth referred to as pro II barnase (7). When *B. amyloliquefaciens* is grown at 20°C, a larger probarnase, designated pro I barnase, is also observed; the amount of pro I barnase is increased in the protease-deficient mutant H2P1 (5) and by the presence of phenylmethylsulfonyl fluoride (PMSF) (Fig. 3). Attempts to isolate pro I barnase from *B. amyloliquefaciens* culture supernatant were unsuccessful because of the propensity of pro I barnase to degrade to pro II barnase. To determine the N-terminal sequence of pro I barnase, we introduced the cloned barnase gene into *B. subtilis* and isolated radiolabeled pro I barnase for microsequencing.

B. subtilis containing pMT71 (expressing inactive barnase, His-102 to Gln [8]) or pMT429 (expressing active barnase [3]) was grown in synthetic medium and labeled for 5 min with [^{3}H]lysine in the presence of PMSF (11). Analysis of the immunoprecipitated protein from the supernatant fraction on a 20% polyacrylamide-sodium dodecyl sulfate gel revealed the presence of a single band. This protein was solubilized and subjected to 15 cycles of automated Edman degradation. The results (Fig. 4) show that cycles 2 and 9 were labeled in both pMT71 and pMT429. These results suggest that the primary peptidase cleavage occurs at the Ala-Ala junction shown in Fig. 1.

The translation and processing of barnase is proposed to be as follows. The major translation initiation codon is TTG (Fig. 1, codon −39). Preprobarnase contains a 26-amino-acid signal peptide with a signal peptidase cleavage site at Ala-Ala (Fig. 1) to produce pro I barnase. Pro I barnase is subsequently cleaved proteolytically to yield pro II barnase and mature barnase. That pro I barnase was the major secreted species in the presence of the serine protease inhibitor PMSF suggests that cleavage of pro I barnase to pro II barnase and mature barnase may be mediated by a serine protease.

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