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mRNA Secondary Structure in an Open Reading Frame Reduces Translation Efficiency in *Bacillus subtilis*

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The structural gene for thermostable neutral protease, *nprM*, has only one stacking region, whose energy is −16.3 kcal/mol (−68.2 kJ/mol). Mutations for increasing (−30.8 kcal/mol [−128.9 kJ/mol]) and decreasing (−5.0 kcal/mol [−20.9 kJ/mol]) the energy of the stacking region were introduced in *nprM* on the recombinant plasmid pMK1 by using site-directed mutagenesis without any amino acid substitutions. The resultant plasmids were designated pMK2 and pMK3, respectively. The enzyme productivity of the pMK2 carrier was about 40% lower than that of pMK1, whereas the productivity of the pMK3 carrier was about 5% higher. The higher the stability of the stacking regions, the lower the enzyme productivity that was observed. mRNA concentrations were almost the same in the cells harboring these three plasmids. These results indicate that the secondary structure of mRNA reduces the translation efficiency.

Two thermostable neutral protease genes, *nprM* and *nprT*, from *Bacillus stearothermophilus* have been cloned in *Bacillus subtilis*, and the nucleotide sequences have also been determined (5, 9, 16). Their enzymes, NprM and NprT, were more and less thermostable than the well-known thermostable neutral protease, thermolysin, respectively (8, 10, 11). To compare the enzyme productivity of *nprM* and *nprT*, which have almost the same promoter sequence, we subcloned these genes on the same copy number vector plasmid, pTB51, in *B. subtilis*. The enzyme productivity of *nprM* was about 20 times higher than that of *nprT* (9), even though the genes had the same copy number and almost the same promoter sequence. It is well known that the secondary structure of mRNA affects its stability (2, 3, 19) and gene expression (6, 15, 20). To analyze the relationships between the mRNA secondary structure in an open reading frame and gene expression, we compared the characteristics of these structural genes.

The amino acid homology of the two proteases was high (about 85%), whereas the G+C contents of *nprT* and *nprM* were 58 and 42 mol%, respectively (9, 16). By using an NEC PC-9801 computer (Nippon Electric Co., Tokyo, Japan) and the GENIAS system (Mitui Information, Tokyo, Japan), we searched the stacking region in *nprM* and *nprT* genes under the following conditions: minimum stacking length, 6 base pairs (bp); maximum stacking energy, −15 kcal/mol (−63 kJ/mol) (17); maximum loop-out, 25 bp (Table 1). The result shows that the eight stacking regions were found in *nprT*, whereas only one stacking region was detected in *nprM*. The high G+C content of *nprT* might stimulate the formation of stable stacking regions. These data suggested that the stacking region might reduce the level of gene expression, including transcription, mRNA stability, and translation.

To clarify this point, we attempted to change the energy of the stacking region in *nprM*. The recombinant plasmid containing *nprM* was designated pMK1, and the structure is shown in Fig. 1. The mutations for increasing and decreasing the energy of the stacking region were introduced into the *nprM* structural gene on pMK1 by using site-directed mutagenesis without any amino acid substitutions (Fig. 2). The *Hin*dIII-*Sph*I DNA fragment (272 bp) of the *nprM* gene (Fig. 1) was subcloned into bacteriophage M13mp18 (12), and the mutations were introduced by using chemically synthesized oligonucleotides (5′-AGCTATGAC*GC*ACCAGCGGT-3′ [Ser TyrAspAlaProAla] for pMK2 and 5′-GATGCTCC*TGC*AG TTGATGC-3′* [AspAlaProAlaValAsp] for pMK3, where asterisks indicate mismatches and underlined sequences are the new restriction sites, *Hga*I and *Pst*I, respectively) with an oligonucleotide-directed in vitro mutagenesis system (Amersham Corp., Amersham, England). The mutations were confirmed by DNA sequencing.

The resultant plasmids, pMK2 and pMK3, and the wild-type plasmid, pMK1, were used to transform *B. subtilis* MT-2 (9). The competent cells were prepared as described previously (7). These transformants were cultivated in 100 ml of L broth (1% Trypton, 0.5% yeast extract, 0.5% NaCl [pH 7.2]) at 37°C for 24 h, and the cell growth and enzyme productivity were measured. No significant differences in

TABLE 1. Stacking region in *nprM* and *nprT*

Enzyme	Position[a]	Sequence no.[b]	Sequence	Energy (kJ/mol)
NprM	56	913–918	CCAGCG	−68.2
		941–936	GGTCGC	
NprT	4	104–109	CGCCGA	−72.0
		123–118	GCGGCT	
NprT	18	320–331	ATACCGTGATGC	−117.2
		356–345	TATGGCACTACG	
NprT	41	710–715	GCCGCC	−88.7
		726–721	CGGCGG	
NprT	42	732–739	GCCGGTCG	−75.3
		759–752	CGGCTGGC	
NprT	56	958–963	GACGCC	−68.2
		981–976	CTGCGG	
NprT	66	1109–1115	ACGGCGA	−66.1
		1141–1135	TGCCGTT	
NprT	75	1281–1286	CGCCAA	−64.4
		1312–1307	GCGGTT	
NprT	80	1334–1340	TCGCCGG	−92.0
		1372–1366	AGCGGCC	

[a] The total length (pre, pro, and mature regions) is taken as 100.
[b] For nucleotide sequences of *nprM* and *nprT*, see references 9 and 16, respectively. The 5′→3′ sequence is given above the 3′→5′ sequence.

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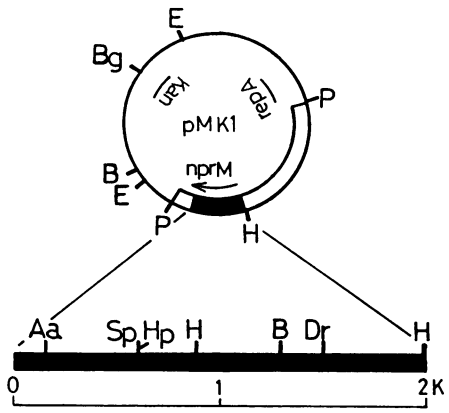

FIG. 1. Structure of plasmid pMK1. Symbols: □, ■, DNA from *B. stearothermophilus* MK232; ←, ■, position and direction of the *nprM* gene. Cleavage sites of *Aat*I, *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Hin*dIII, *Hpa*I, *Pst*I, and *Sph*I are indicated by Aa, B, Bg, Dr, E, H, Hp, P, and Sp, respectively. Abbreviations: Kan, kanamycin resistance gene; repA, replicon of the plasmid.

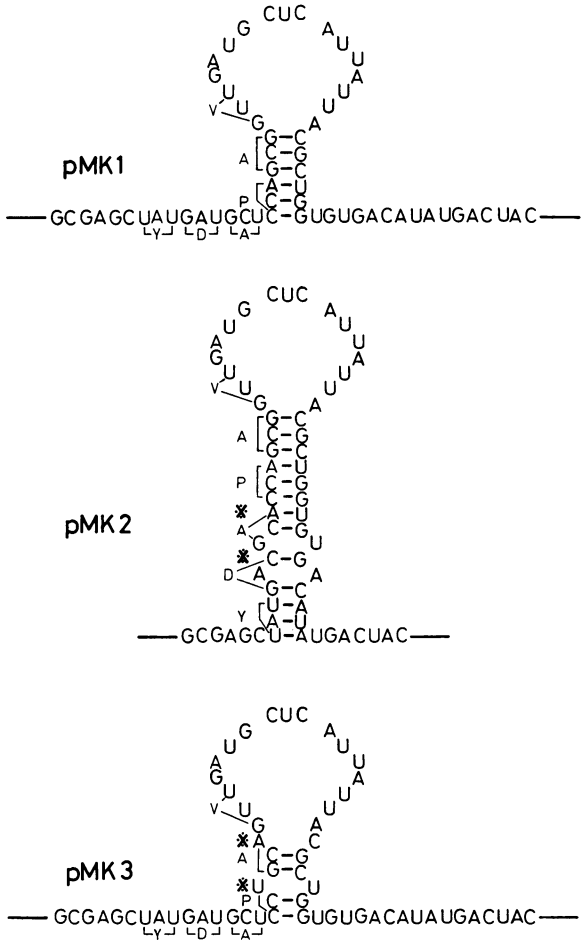

FIG. 2. Possible mRNA secondary structure and corresponding thermodynamic stabilities of *nprM* mRNA from pMK1 (wild type), pMK2 (site-directed mutation), and pMK3 (site-directed mutation). Asterisks indicate the positions of mutation. The stacking energies were calculated by the method of Tinoco et al. (17) and are as follows: pMK1, −16.3 kcal/mol (−68.2 kJ/mol); pMK2, −30.8 kcal/mol (−128.9 kJ/mol); pMK3, −5.0 kcal/mol (−20.9 kJ/mol). Amino acid codons are indicated by single letters: A, Ala; D, Asp, P, Pro; V, Val; Y, Tyr.

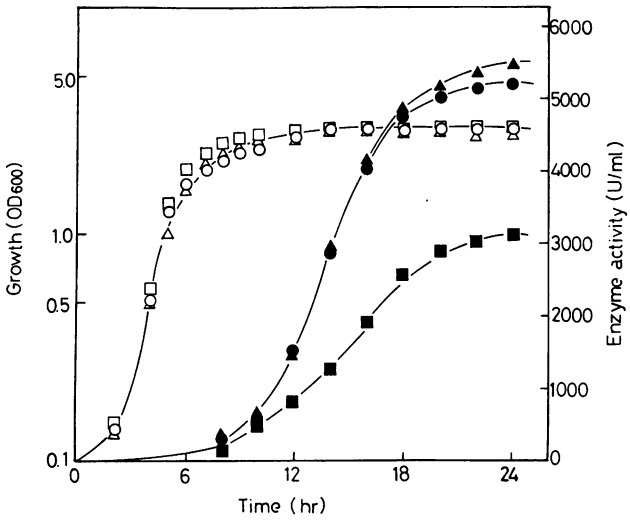

FIG. 3. Time course of neutral protease production and cell growth of *B. subtilis* MT-2 carrying pMK1, pMK2, and pMK3. Cells were grown on a rotary shaker at 37°C in L broth containing 5 μg of kanamycin per ml. The culture supernatants were used for the enzyme assay (10) (●, pMK1; ■, pMK2; ▲, pMK3). Bacterial growth (○, pMK1; □, pMK2; △, pMK3) was measured by monitoring the optical density at 660 nm.

cell growth between the three strains were found (Fig. 3). The enzyme productivity of the pMK2 carrier was about 40% lower than that of the pMK1 carrier. In contrast, the productivity of the pMK3 carrier was about 5% higher than that of the pMK1 carrier. These data, together with comparisons between *nprM* and *nprT* genes, indicate that the stacking region in the structural gene tended to reduce the enzyme productivity.

It is generally believed that the enzyme productivity of recombinant plasmid carriers is influenced by the plasmid copy number and the strength of the promoter. However, it is very interesting that the stacking regions in the open reading frame of a structural gene regulated the enzyme productivity. The relationship between the stacking region and the enzyme productivity might be explained by the following possibilities: (i) when mRNA is transcribed, the stacking region might function as a terminator; or (ii) the stacking region might make a stable mRNA secondary structure, which might inhibit efficient translation.

To understand the role of the stacking region in the structural gene, we measured the quantity of transcribed mRNA by Northern (RNA) blot analysis (Fig. 4). Total RNA was prepared by the method of Duvall et al. (4) and Shimotsu et al. (14). The *Bam*HI-*Aat*I fragment (1,210 bp) of pMK1 (Fig. 1) was labeled by using the Takara random DNA-labeling kit (Takara Shuzo Co., Kyoto, Japan) and used as a probe. The Northern blot analysis was performed as described by Alwine et al. (1) with Hybond-N hybridization transfer membranes (Amersham). The results indicate that the amount and size of the specific mRNA in the cells harboring pMK1, pMK2, and pMK3 were nearly the same, although the transcription rate and the stability of the mRNA were not separately measured. It is therefore clear that the

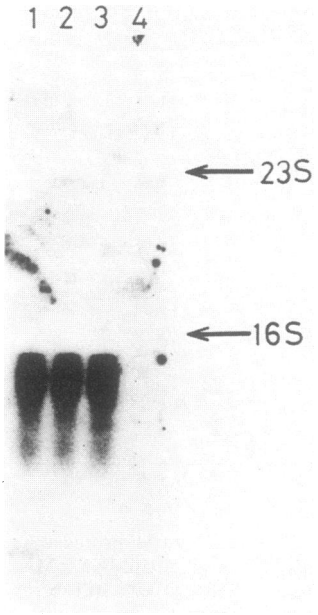

FIG. 4. Northern blot analysis of *nprM* mRNA from *B. subtilis*. A 1,210-bp *Bam*HI-*Hpa*I DNA fragment within the *nprM* gene was obtained from pMK1 and used as the hybridization probe. Lanes: 1, RNA from *B. subtilis* MT-2(pMK1) cells grown for 24 h; 2, RNA from *B. subtilis* MT-2(pMK2) cells grown for 24 h; 3, RNA from *B. subtilis* MT-2(pMK3) cells grown for 24 h; 4, RNA from *B. subtilis* MT-2(pTB51) (vector plasmid) cells grown for 24 h. The positions of 16S and 23S rRNAs are shown.

mRNA level is not affected by the stacking region. Although the same quantity of mRNA is transcribed from the three plasmids, the enzyme productivities were quite different. Although four codons were changed to synonymous codons (GAU to GAC, GCU to GCA, CCA to CCU, and GCG to GCA) in this study, all these codons are frequently used in *B. subtilis* (13). Therefore, it is most unlikely that the codon usage significantly affects the translation efficiency. From these observations and discussion, we concluded that the secondary structure within the mRNA coding region could regulate the translation efficiency; i.e., when the free energy of the stem-loop structure increased, the secondary structure of mRNA became more stable and the translation efficiency decreased. The same idea has also been reported for eucaryotic mRNA (18). Accordingly, if the stable secondary structure of mRNA were artificially eliminated, higher enzyme productivity may be possible.

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