Note

Nucleotide Sequence of the Gene Increasing the Extracellular Proteolytic Activities of *Bacillus licheniformis*; Comparison with Similar Phenotypic Genes from Other *Bacillus* sp.

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Secretion of enzymes by microbial cells has been attracting increased interest recently, not only as to elucidation of the secretion mechanism, but also as to the creation of microbial cells having high ability to produce enzymes extracellularly. Among various enzymes, the proteases of *Bacillus* sp. are the most typical and useful extracellular enzymes, and have been regarded as a model system for studying the secretion mechanisms for enzymes. So far, we have reported the cloning of a gene responsible for the secretion of alkaline and neutral proteases by *Bacillus licheniformis*.¹⁾ However, the gene was not long enough to encode both types of proteases and, therefore, our interest was focused on how the cloned gene stimulates the protease secretion. Recently, similar phenotypic genes having the ability to increase protease secretion were cloned from *Bacillus amyloliquefaciens*,²⁾ *Bacillus natto*³⁾ and *Bacillus subtilis*.⁴⁾ Comparison of the nucleotide sequences of these genes revealed that the genes from *B. amyloliquefaciens* and *B. subtilis* showed significant homology, while the gene from *B. natto* was different from the other two genes. So, we determined the nucleotide sequence of the gene from *B. licheniformis* in order to compare it with those of the genes from other *Bacillus* sp.

B. subtilis 1012 (*leuA8 metB5 nonB hsrM1*)⁵⁾ was used as a host for vector plasmid pUB110. *Escherichia coli* JM109 was used for sequencing. The gene responsible for the extracellular proteolytic activities of *B. subtilis* was first cloned from *B. licheniformis* onto vector plasmid pUB110 and the resulting hybrid plasmid was designated as pAN2.¹⁾ The gene was then subcloned into the *Bam*HI site of vector plasmid pUB110 as a *Sau*3AI partially digested

fragment [ca. 320 base pairs (bp)]. The most truncated hybrid plasmid, designated as pAN2-2, was used for sequencing. The *Xba*I-*Pvu*II fragment with the gene from *B. licheniformis* was recloned at the *Xba*I/*Hinc*II site of vector plasmid mp18 [Fig. 1(A)]. Two *Sau*3AI fragments constituting the fragment cloned from *B. licheniformis* were confirmed by reading through of the sequence from the *Xba*I site to the *Pvu*II site. Sequencing was also performed for two *Sau*3AI fragments, individually, in different directions. The sequencing was performed by the chain termination method according to the vendor's specifications (Takara Shuzo Co., Ltd., Kyoto, Japan).

The complete nucleotide sequence of the gene that promoted protease secretion is shown in Fig. 1(B). The subcloned fragment in pAN2-2 was composed of two *Sau*3AI fragments containing 132 bp and 186 bp, respectively. Comparison of the nucleotide sequence of the two *Sau*3AI fragments with those of the genes from *B. amyloliquefaciens*, *B. subtilis* and *B. natto* revealed that the shorter fragment (+1~+132) showed more than 80% homology with the genes from *B. amyloliquefaciens* and *B. subtilis*, although the longer fragment (+129~+314) contained no significant common sequence with the other three genes mentioned above. Since, in *B. amyloliquefaciens* and *B. subtilis*, the homologous *Sau*3AI fragment has been shown to encode a short polypeptide of 46 amino acids, the shorter *Sau*3AI fragment from *B. licheniformis* was also expected to encode such a short polypeptide. The results of the translation experiment indicated that the *Sau*3AI fragment encoded a polypeptide, and the region translating the short polypeptide started at +27 and stopped at +146, i.e., spanning the longer *Sau*3AI fragment. This open reading frame translated 40 amino acids accompanied two tentative Shine-Dalgarno sequences upstream of the initiation codon. Furthermore, the promoter region for transcription is presumably located in the segment between the *Xba*I and *Bam*HI/

FIG. 1(A). Restriction Map and Sequencing Strategy for the Gene Responsible for Extracellular Proteolytic Activities of *B. licheniformis*.

The length and direction of the sequencing with the phage clone is shown with an arrow. The putative region coding 40 amino acids is also depicted, with a thick arrow. ■, the fragment derived from vector plasmid pUB110; □, the fragment cloned from *B. licheniformis*.

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Xba I -----TTGATTACAAC

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-120      GAACGTGTGCAAAAGAAATATGATTAGAATCACAAAAAACAGGTACAAGAAAAACGAGGAAA
-90
-60      GATGCTGTTCTTTGTAAATGAGTTGCTAGTAACATCTGACCGAGATTTTTTTGAGCAACTG
-30      +1
+1      GATCTTCCAAAAGGAGTGTGGAACCGGTGGAAAAAGCAACAAATTGAAGAATTAAAAACAAC      +60
MetGluLysGlnGlnIleGluGluLeuLysGlnLeu
1
+30
+90      TGCTTTGGCGGCTAGAGAATGAAATCAGAGAAACAAAGGACTCCTTGCGCAAGATTAACA
+120
LeuTrpArgLeuGluAsnGluIleArgGluThrLysAspSerLeuArgLysIleAsnLys
13
+150
AAAGCATTGATCTGTTAATACAAAGTTTAAAGGGATTTGGTTTCTTTTCACCGAAAAGTT      +180
SerIleAspLeuLeuIleGlnVal***
33      40
+210
GCATATAAATTATGCGTTACACCGCAAACCTTTAAATAGACCATAACACCCATTTTACAC      +240
+270
AACTTGCGATTAGTTCTCAAACTCTAATGAAACATAAAATTTTTGTTGTTTTTCAAGGA      +300
+314
GTCAAATAAAGATC

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FIG. 1(B). Nucleotide Sequence and Deduced Amino Acid Sequence of the Gene Responsible for Extracellular Proteolytic Activities of *B. licheniformis*.

The nucleotide sequence is numbered starting at the *Sau3AI* site (GATC) formed on insertion of the gene cloned from *B. licheniformis* into the *BamHI* site of vector plasmid pUB110.

The Shine–Dalgarno sequence, “–10 region” and “–35 region” are underlined with solid, dotted and waved lines, respectively. *Sau3AI* sites of the gene are boxed.

Lich. 1 Met Glu Lys Gln Gln Ile Glu Glu Leu Lys Gln Leu Leu Trp Arg Leu Glu Asn Glu Ile 20
 Amyl. 1 Met Glu Lys Lys Leu - Glu Glu Val Lys Gln Leu Leu Phe Arg Leu Glu Asn Asp Ile 19
 Subt. 1 Met Glu Lys Lys Leu - Glu Glu Val Lys Gln Leu Leu Phe Arg Leu Glu Leu Asp Ile 19

21 Arg Glu Thr Lys Asp Ser Leu Arg Lys Ile Asn Lys Ser Ile Asp Leu Leu Ile Gln Val 40
 20 Arg Glu Thr Thr Asp Ser Leu Arg Asn Ile Asn Lys Ser Ile Asp Gln Leu Asp Lys Phe 39
 20 Arg Glu Thr Thr Asp Ser Leu Arg Asn Ile Asn Lys Ser Ile Asp Gln Leu Asp Lys Tyr 39

40 Ser Tyr Ala Met Lys Ile Ser 46
 40 Asn Tyr Ala Met Lys Ile Ser 46

FIG. 2. Comparison of the Amino Acid Sequences of Putative Polypeptides Encoded by the Genes Promoting the Extracellular Protease Activities of *B. licheniformis* (Lich.), *B. amyloliquefaciens* (Amyl.) and *B. subtilis* (Subt.).

Amino acid residues differing from each other are boxed.

Sau3AI sites of the vector plasmid, pUB110, because the region was so A-T rich as to easily separate into single strands. Particular, two consensus promoter sequences, “-35 and -10 regions” (TTGTAA and TAACAT), were conserved at positions -50 and -33, respectively.

with spacing of 12 bp. Besides these hexanucleotides, many candidates, which matched the two conserved sequences, as promoters were found in the upstream region. But there was no pair of “-35 and -10 regions” meeting the requirement of an appropriate distance.

Therefore, the *B. licheniformis* gene probably uses the vicinity of the *Bam*HI site of vector plasmid pUB110 as a transcriptional promoter in order to be expressed. Moreover, a consecutive A and T structure, which was found to be functional as a transcriptional terminator, was apparently present downstream of the translational termination codon, in spite of that no remarkable stem-loop structure existed.

The polypeptide translated by the region highly conserved among these *Bacillus* sp. are compared in Fig. 2. The amino acid sequence of the polypeptide encoded by the *B. licheniformis* gene showed 70% homology with that in the cases of the *B. amyloliquefaciens* and *B. subtilis* genes. This suggested that, although the length of the polypeptide of *B. licheniformis* was slightly shorter by 6 amino acids than those of the others, such polypeptides may have the same function in *Bacillus* cells. Although there is an almost complete lack of evidence as to the functions of such polypeptides, two possibilities have been discussed. One is the stimulation of protease excretion across the cell membrane, and the other is the acceleration of transcription of mRNA for proteases.⁶⁾ The observation of Young *et al.*⁴⁾ that β -galactosidase fused with *B. subtilis* subtilisin was cytoplasmically increased by the gene responsible for the

protease secretion of *B. subtilis* may support the latter explanation. Further study is now required for elucidation of the intrinsic function of the polypeptide.

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