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NUCLEOTIDE SEQUENCE OF THE PROMOTOR AND NH_2-TERMINAL SIGNAL PEPTIDE REGION OF *Bacillus subtilis* α-AMYLASE GENE CLONED IN pUB110

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SUMMARY The nucleotide sequence of the promotor and NH_2-terminal signal peptide region of the α-amylase gene derived from the α-amylase hyper-producing strain *B.subtilis* NA64 was determined. DNA sequences of the NH_2-terminal region of the mature α-amylase, 41 amino acid residues of the signal peptide, a Shine-Dalgarno sequence (AGGAG), a potential RNA polymerase recognition site (TTGAAA), and a potential Pribnow box (AAGTAA) were identified. The DNA sequence was quite different from that of the α-amylase gene of *B.amyloliquefaciens*.

INTRODUCTION Extracellular enzymes contain NH_2-terminal amino acid extensions, "the signal peptide", which are subsequently removed by a specific peptidase resulting in the mature enzymes (1,2). The signal peptide and peptidase mechanism of secretion of extracellular proteins seems to be similar in both prokaryotic and eukaryotic cells (3).

A large number of proteins in the periplasmic space and the outer membrane have been visualized in *E.coli* in the presence of signal peptide (1). On the other hand, *B.subtilis* is known to secrete a large number of soluble proteins into the culture medium, but detailed information and signal sequences of the secreted proteins have not been reported. Of the gram-positive bacilli, penicillinase of *B.licheniformis*

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and α-amylase of B.amyloliquefaciens were shown to have 34 and 31 amino acid residues in their signal peptides, respectively (4,5,6). The production of α-amylase has been the subject of many biochemical and genetical studies (7). In our laboratories we have constructed an ultrahyper α-amylase producing strain, T2N26, into which has been placed at least six kinds of regulatory genes. It produces 1-2 x 10^3 times as much α-amylase as the original strain, B.subtilis 6160, did (8). We cloned the α-amylase gene (amyR2 amyE$^+$) in plasmid pUB110 in order to apply this highly active protein secretion system to gene engineering. Herein we describe the nucleotide sequence for the promotor and the NH_2-terminal amino acid region of the gene.

MATERIALS AND METHODS The α-amylase producing plasmid pTUB4:pTUB4 was constructed by inserting a 2.3 Kb fragment (amyR2 amyE$^+$) which was derived from the α-amylase hyper producing strain B.subtilis NA64 into the plasmid pUB110. DNA from a specialized transducing phage ρ11damyE$^+$-aroI$^+$ was ligated to BamHI-cleaved pUB110 after having been partially digested by the restriction enzyme Sau3A. The plasmid was then transferred into B.subtilis 207-25 (hsdM recE4 amyE07 aroI906 metB5 leuA8 lys21). The transformants which harbored pTUB4 produced 20 - 40 times as much α-amylase as NA64 did (9).

Agarose and polyacrylamide gel electrophoresis:Large molecular size DNA fragments were separated by agarose gel (0.6 - 1.5%) electrophoresis and small DNA fragments by 5% polyacrylamide gel electrophoresis. For preparative purpose, the DNA fragments were electroeluted from agarose into hydroxyapatite (10).

Enzymes:Restriction enzymes and polynucleotide kinase were purchased from Takara Shuzo Co. Ltd.(Kyoto, Japan) and from Bethesda Research Laboratories, Inc. (Maryland, USA). Each was used according to the manufacturer's specification.

DNA sequence determination:DNA fragments to be sequenced were eluted from gels and labeled at 5' end with [γ-^{32}P] ATP and polynucleotide kinase. DNA sequences were determined by the method of Maxam and Gilbert (11). The cleaved products were separated on 8% or 20% polyacrylamide gels containing 8.3 M urea. The gels were autoradiographed at -70°C using intensfying screen (Du pont).

RESULTS The order of restriction sites in the inserted DNA from the XbaI site to the BglII site was ClaI, EcoRI, PstI, and SalI (Fig.1). The EcoRI site was located at near the center of the inseted DNA. The gene order around amyE on the B.subtilis chromosome was tmrA-amyR-amyE-tmrB-aroI (12). When ρ11damyE$^+$aroI$^+$ genome was included the genes in this order, the restriction sites maintained the same order (ClaI-SalI)

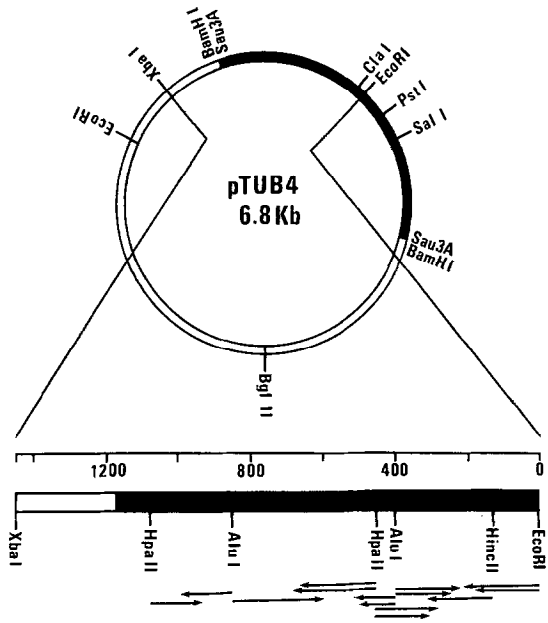

Fig.1. Physical map of plasmid pTUB4 carrying α-amylase structural gene (*amyE*$^+$) and a specific regulatory gene (*amyR2*) derived from an α-amylase hyper-producing strain, B.subtilis NA64. The NH_2-terminal region of the α-amylase gene in pTUB4 (XbaI-EcoRI-1.45 Kb) was purified by agarose gel electrophoresis after pTUB4 was digested in a mixture of the three restriction enzymes, EcoRI, XbaI and BglII. The physical map was studied using restriction enzymes. The sequence strategy of the NH_2-terminal region of *amyE*$^+$ in pTUB4 is shown at the bottom. The 2.3 Kb fragment (■) was inserted into *B.subtilis* plasmid vector pUB110 (□).

around *amyE*$^+$ (K.Yamane *et al.*, unpublished). These results suggest that the DNA fragment from the XbaI site to the EcoRI site of pTUB4, approximately 1.45 Kb, contains a regulatory gene, *amyR2*, and the NH_2-terminal region of *amyE*$^+$. Although we analysed a DNA sequence of about 800 bp upstream from the EcoRI site presenting a detailed restriction site map of the promotor and NH_2-terminal region (fig.1), we are showing 300 bp long of the nucleotide sequences of the specific promotor-NH_2-terminal region (Fig.2).

The amino acid sequence of the NH_2-terminal region of α-amylase of *B.subtilis* YY88 was determined by Mäntsälä and Zalkin (13). YY88 contained the same α-amylase structural gene (*amyE*$^+$) used in this experiment, because it is a transformant of strain NA64 into which had been introduced a regulatory gene, *pap9*, by DNA mediated transformation

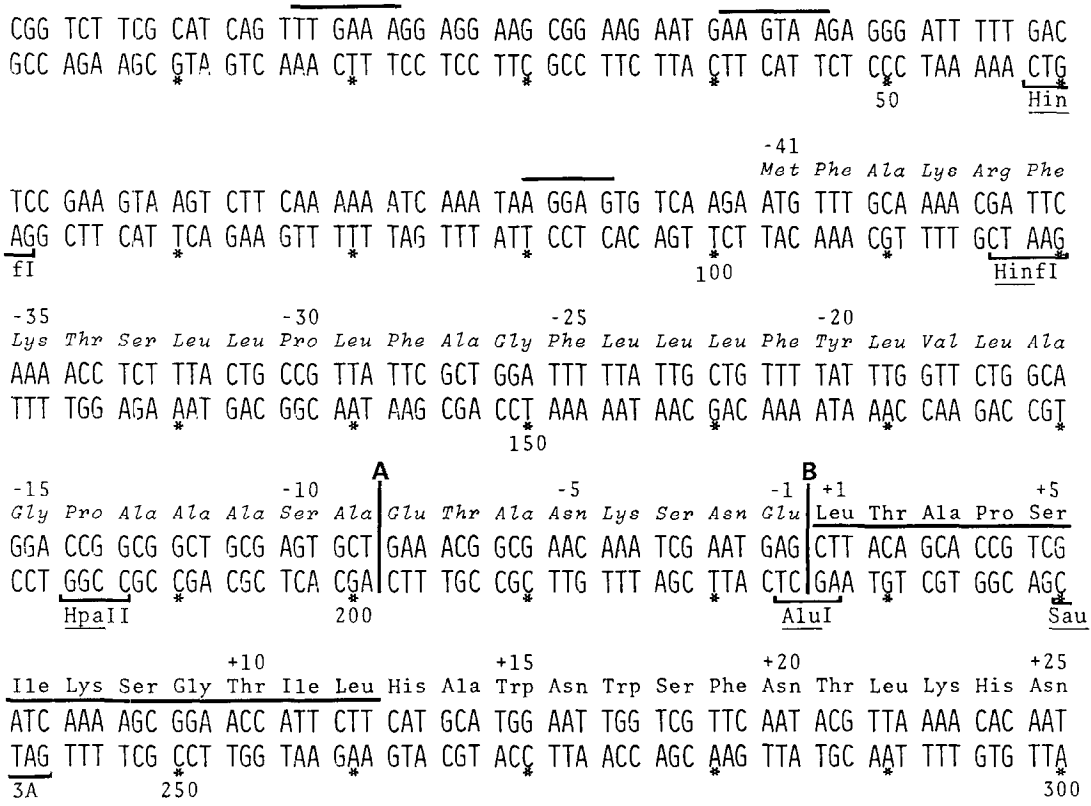

Fig.2. DNA and amino acid sequence in the NH_2-terminal region of B.subtilis α-amylase gene. The NH_2-terminal leucine of the mature α-amylase was taken as amino acid +1. The cleavage site between the signal sequence and the mature α-amylase is indicated by a verical bar (B), and a possible first cleavage site by another vertical bar (A). The signal sequence (amino acids -1 to -41) is shown in italics. Published amino acid sequence is underlined. The potential RNA polymerase recognition site (TTGAAA), Pribnow box (AAGTAA) and Shine-Dalgarno sequence (AGGAG) are indicated by solid lines.

(14). The amino acid sequence deduced from the DNA sequence (nucleotides 226 - 261) is identical to the sequence of 12 amino acid residues (Leu -Thr-Ala-Pro-Ser-Ile-Lys-Ser-Gly-Thr-Ile-Leu) reported.

There is only one initiator ATG codon located at position 103-105 in the phase of the reading frame of the structural gene. This suggests that the B.subtilis α-amylase secreted into the medium is derived from a procursor molecule containing 41 amino acid residues which correspond to the signal peptide for the α-amylase. The 5' flanking region of nucleotide 1 - 102 contains typical sequences for the regulatory signals

of gene expression in prokaryotes. Nucleotides 90 - 94 (AGGAG), a Shine-Dalgarno sequence for translation initiation, are found 8 nucleotides upstream of the ATG codon. The potential RNA polymerase recognition site (TTGAAA) and the potential Pribnow box (AAGTAA) are found at nucleotides 17 - 22 and 41 - 46, respectively. This seems to be a typical B.subtilis promotor region (15).

DISCUSSION It is clear that there is only one potential initinator, the ATG (103 - 105) codon, located at the amino acid position -41 which precedes the NH_2-terminal Leu of the mature α-amylase. This strongly confirms the idea that B.subtilis α-amylase is synthesized as a precursor protein with an NH_2-terminal peptide extension as in the penicillinase of B.licheniformis, α-amylase of B.amyloliquefaciens and others (1,4,5,6). Within the α-amylase signal sequence, there is a stretch of 24 uncharged mainly hydrophobic amino acids as in other known signal peptides(1). Furthermore, there is a cluster of charged amino acids, one Arg, and two Lys residues, between the NH_2-terminal Met residue and the hydrophobic region. However the signal sequence of B.subtilis α-amylase is strikingly long (41 amino acid residues) compared to most other exported proteins of prokaryotes and eukaryotes (1). Many signal sequences in precursor proteins have been cleaved between Ala and X amino acid to make mature proteins. In particular, the precursors of α-amylase from B.amyloliquefaciens (6) and membrane penicillinase of S.aureus (5) were cleaved between Ser-Ala and X amino acid. Therefore, it is possible that B.subtilis α-amylase will be first cleaved between Ser-Ala and Glu at the amino acid position -9 — -8, and subsequently between Glu and Leu as in the formation of exo-penicillinase of B.licheniformis and S.aureus (5).

Starting from nucleotide 226, an amino acid sequence can be deduced which completely agrees with the 12 amino acid sequence of the NH_2-terminal region of YY88 α-amylase. When the sequence of YY88 α-amylase is compared with that of the NH_2-terminal region of α-amylase from B.

subtilis var. amylosaccharitícus (SAC α-amylase), there is clearly extensive homology between the two saccharifying type α-amylases. They are different at the NH_2-terminus itself, but a 2nd amino acid residue (Thr) of YY88 α-amylase is identical to the terminus of SAC α-amylase. Other 9/10 amino acid residues of the former were identical to those of the latter (13, 16). However these amino acid sequences of the B.subtilis α-amylases were completely different from those of liquefying type α-amylase from B.amyloliquefaciens (6).

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