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MODULATION OF Bacillus subtilis α-AMYLASE PROMOTER ACTIVITY BY THE PRESENCE OF A PALINDROMIC SEQUENCE IN FRONT OF THE GENE

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Upstream of the promoter of the Bacillus subtilis α-amylase gene (amyE) derived from an α-amylase hyper-producing strain, there is an inverted repeat sequence (palindromic sequence), which has a free energy of 21.2 kcal/mol due to the formation of stable stem-loop structure. The role of the palindromic sequence for the expression of amyE was studied using a plasmid encoding the amyE'-'bla (E. coli β-lactamase) fused gene in an α-amylase-deficient B. subtilis mutant as the host. By the presence of the palindromic sequence, the transcription activity of the amyE promoter was enhanced approximately 6 fold by starch (3 %) in the medium and was less repressed by glucose.

α-Amylase is one of the major extracellular enzymes of Bacillus subtilis. The expression of the gene (amyE) for the α-amylase is controlled by a regulatory gene (amyR), which is located close to the amyE gene (1,2). We cloned amyR2 and amyE from the chromosomal DNA of an α-amylase hyper-producing strain, B. subtilis NA64. The DNA nucleotide sequence in the amyR2 region revealed that there was an inverted repeat sequence (palindromic sequence) upstream of the B. subtilis α-amylase promoter, which has a free energy of 21.2 kcal/mol due to the formation of a stable palindromic structure (3,4). In contrast, the palindromic sequence was not recognized in the sequence of the amyR1 region of B. subtilis Marburg 168 (Fig.1)(5). The promoter activity of amyR1 was repressed by glucose in the culture medium (6).

In order to clarify the role of the palindromic sequence in the expression of amyE, we constructed a B. subtilis plasmid pTUB257, in

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which the structural gene (bla) for the E. coli β-lactamase was fused downstream of the DNA region coding for the signal peptide of amyE. Then the palindromic sequence in amyR2 of pTUB257 was eliminated. The expression of amyE was deduced from the formation of extracellular β-lactamase using an α-amylase-deficient B. subtilis mutant as the host. By the presence of the palindromic sequence, the transcription of the gene was activated by the addition of starch in the culture medium and was partially repressed by glucose.

MATERIALS AND METHODS

Bacterial strain and plasmids : The α-amylase-deficient strain of Bacillus subtilis 207-25 is a derivative of B. subtilis 168 (3). The B. subtilis plasmid pTUB257 is a derivative of pTUB256, in which the bla gene was introduced downstream of the B. subtilis α-amylase promoter and the 33 amino acid-signal peptide coding regions using a B. subtilis vector pUB110 (Fig. 2-a)(7). Upstream of the promoter region (-152 ∼ -124) of pTUB257, there is the palindromic sequence (-273 ∼ -225), which is rich in A and T (Fig. 1).The EcoRI-AvaI 99 bp DNA fragment (-302 ∼ -204)was eliminated from pTUB257. The constructed plasmid was designated as pTUB258 (Fig. 2-b). Transformation of B. subtilis 207-25 by the plasmids was performed by the protoplast transformation method of Chang and Cohen (8).

Assay of β-lactamase activity : β-Lactamase activity in the culture media and cells was measured by the method of O'Callagham et al (9). One unit of β-lactamase hydrolyzed one n mol on nitrocefin (BBR Biological Systems, Maryland, USA) in one min at 37°C.

RNA dot blot hybridization : RNAs from B. subtilis cells were prepared by the hot phenol method of Shimotsu et al (10). Denatured RNAs were dot blotted onto nitrocellulose filters (Schleicher & Shuell BA85) with a manifold apparatus, as described by Wahl et al (11). To prepare

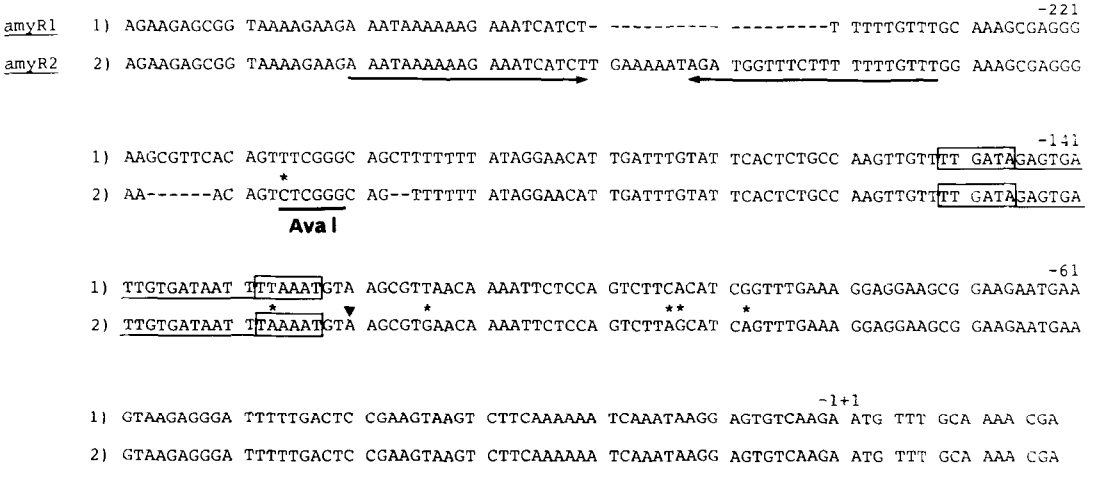

Fig. 1. Comparison of DNA nucleotide sequence of the amyR1 (5) and amyR2 (3) regions. * , difference in base pairs between amyR1 and amyR2; ⟶ ⟵ palindromic sequence found in amyR2; - , gaps in the optimal sequence alignment; ⊓ ⊓ , promoter regions; ▲, transcription initiation sites.

^{32}P-labeled single stranded nonsense DNA for the probe, the HindIII-PstI 480 bp DNA fragment of bla of pTUB256 was inserted into M13 mp10. Sense strand DNA was prepared. The M13 phage DNA was labeled with [α-^{32}P] dCTP by extension of the annealed sequence primer with DNA polymerase I (Klenow fragment) at 15°C for 1 h. RNA-DNA hybridization and radioautography were performed by the method of Thomas (12).

Assay of relative copy numbers of plasmid : B. subtilis 207-25 harboring pTUB257 (pTUB257-strain) and that harboring pTUB258 (pTUB258-strain) were cultured for 5 or 9 hs. The cells were precipitated by centrifugation at 14,000 rpm for 5 min, suspended and adjusted to a A_{660} of 8.00 in 50 mM TrisHCl buffer, pH 8.0 containing 10 mM EDTA. Plasmids were extracted from 1 ml of the cell suspensions by the rapid alkaline method of Birnboim and Doly (13), subjected to agarose gel electrophoresis after treatment with RNase, and analyzed by Southern hybridization (14). ^{32}P-labeled pTUB257 and pTUB258 for use as the probes were prepared with a nick translation kit (Amersham International plc, Amersham, England). Relative plasmid copy numbers were measured densitometrically from positive spots in the radioautograms.

RESULTS AND DISCUSSION

The structure of pTUB257 and pTUB258 used in this experiment is illustrated in Fig. 2-a and 2-b. The bla gene in the fused gene was expressed by the B. subtilis α-amylase promoter and the gene product was secreted into the culture medium by the aid of the α-amylase signal peptide as shown in the previous experiment (7). Fig. 2-c, and 2-d indicate the amount of the extracellular β-lactamase produced by the pTUB257- and pTUB258-strains under different culture conditions. The activity curves for the two strains grown on L-broth were quite similar. In contrast, the production of the enzyme by the pTUB257-strain was higher than that by pTUB258-strain in the media containing starch (3 %) or glucose (0.5 %). The cell-bound β-lactamase activity in the three culture media was less than 5 units/ml. Growth curves of the two strains were equal in each culture condition. These results suggest that the palindromic sequence in amyR2 of pTUB257 was responsible for the expression of the fused gene in the presence of starch or glucose in the medium. The decrease in the extracellular β-lactamase activity after 7 h of cultivation denoted in Fig. 2-c and -d was due to the activity of extracellular proteases, which were produced by the host cells (15,16).

In order to confirm that the palindromic sequence modulates the promoter activity, we measured the mRNA contents for the bla gene and the relative plasmid copy numbers. Total RNAs were extracted from the pTUB257- and pTUB258-strains in the mid log phase (5 h cultivation) and

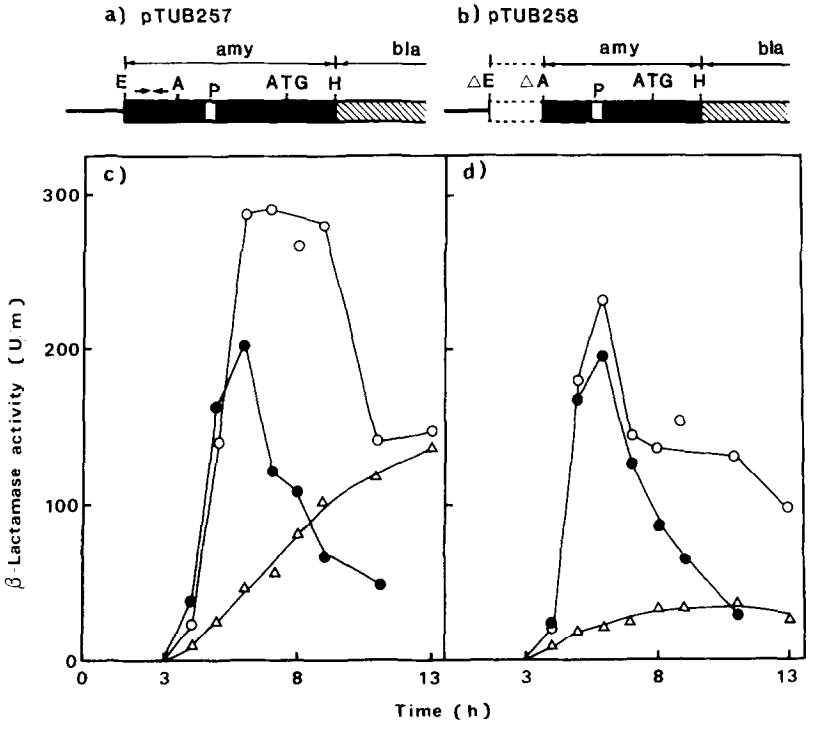

Fig. 2. Structures of the promoters and amyE'-'bla fused gene region of pTUB257 (a) and pTUB258 (b), and extracellular production of E. coli β-lactamase by B. subtilis 207-25 harboring pTUB257 (c) or pTUB258 (d) under different culture conditions. ■, amyR2 amyE region; ▨, bla region; P, promoter region; → ←, paiindromic sequence; ATG, translation initiation site. A, AvaI site; E, EcoRI site; H, HindIII site. ΔA, ΔE, broken AvaI and EcoRI sites. Cells were cultured in L-broth containing 10 μg/ml of kanamycin (●), L-broth containing 10 μg/ml of kanamycin and 3 % starch (○), or L-broth containing 10 μg/ml of kanamycin and 0.5 % glucose (△) at 37°C. The culture media were sampled at the time indicated and centrifuged at 14,000 rpm for 3 min.

in the early stationary phase (9 h cultivation) of growth, and the quantity of the bla mRNA was measured by the dot blot hybridization method (Fig. 3). The intensity of the positive spots increased by the addition of starch, especially in the 9 h cells of the pTUB257-strain, while it decreased by the addition of glucose. The intensity of the spots was densitometrically quantified and the results are summarized in Table I. The contents of bla mRNA in the 9 h cells of both strains, which were cultured in L-broth, were 2-3 times higher than those in the 5 h cells. In contrast, the content in the 9 h cells of the pTUB257-strain in the starch medium increased more than 9 fold. This tendency was similar to the α-amylase production of B. subtilis NA64. The production was stimulated in

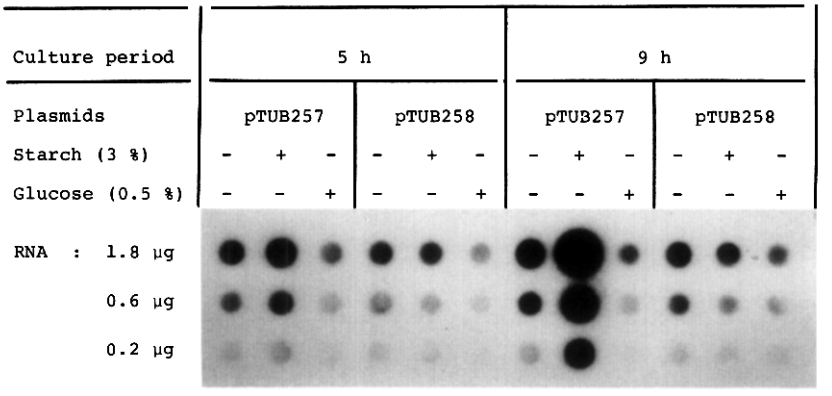

Fig. 3. Dot blot hybridization analysis of bla mRNA. Total RNAs were extracted from the cells after 5 or 9 h of cultivation in the three media. Then a quantity of 1.8 µg, 0.6 µg and 0.2 µg of the total RNAs was blotted in a nitrocellulose filter. Thereafter the bla mRNA was analyzed by the RNA-DNA hybridization method. +, -, addition or no addition of starch or glucose into the L-broth containing 10 µg/ml of kanamycin.

the early stationary phase of growth. Such remarkable increase in the content of bla mRNA was not observed in the pTUB258-strain. The molecular sizes of the bla mRNA from the pTUB257- and pTUB258-strains were identical. To confirm that the increase in the content of bla mRNA in the pTUB257-strain was due to the activation of the promoter, the relative copy numbers of each plasmid was measured as indicated in Table I. The copy numbers increased in the starch medium but decreased by the addition of glucose. The numbers were similar in the cells after 5 and 9 h of cultivation in each medium. Then the transcription activities of the promoters of pTUB257 and pTUB258 were calculated from the relative contents of bla mRNA based on the relative plasmid copy numbers (Table I). The results clearly showed that the promoter in pTUB257 was activated by starch in the medium. These analyses also showed that the promoter activity in pTUB257 was less repressed than that in pTUB258 by the addition of glucose.

During the 13 h period of cultivation of the pTUB257- and pTUB258-strains, starch in the medium was not degraded while glucose was reduced to 0.1-0.15 %. It seems improbable that starch with a higher molecular weight in the medium was incorporated into the cells directly and that the incorporated starch affected the palindromic sequence. The small amount of starch in the medium can be degraded by some enzyme(s) and the degradation

Table I. Comparison of transcription activity of the B. subtilis α-amylase promoters in pTUB257 and pTUB258

Strains	B. subtilis 207-25[pTUB257]						B. subtilis 207-25[pTUB258]					
Culture period	5 h			9 h			5 h			9 h		
Glucose (0.5 %)	-	-	+	-	-	+	-	-	+	-	-	+
Starch (3 %)	-	+	-	-	+	-	-	+	-	-	+	-
Relative content of bla mRNA	1.0	1.3	0.23	2.1	9.1	0.67	1.0	0.52	0.12	1.7	1.8	0.50
Relative plasmid copy numbers	1.0	1.8	0.42	0.96	1.6	0.49	1.0	1.5	0.37	0.82	1.56	0.58
Transcription activity	1.0	0.72	0.54	2.2	5.7	1.4	1.0	0.33	0.31	2.0	1.1	0.86

B. subtilis 207-25 strains harboring pTUB257 and pTUB258 were cultured in three media. The relative content of bla mRNA and relative plasmid copy numbers in the cells were densitometrically analyzed from radioautograms as described in the text. Then the transcription activity of the promoter was estimated.

products or some impurity of starch such as the presence of maltoligosaccharides can be related to the expression of amyE directly or indirectly

The palindromic sequence of amyR2 seemed to be a transcription termination signal for the proximate gene of amyE, because it was located in front of the promoter of amyE. We found, however, that the palindromic sequence also modulated the expression of the amyE gene. The binding site of cAMP-CRP (cAMP receptor protein) in E. coli lac operon is one of the most extensively studied palindromic sequence for the release from glucose repression (17,18). In B. subtilis, such a regulatory system has not been demonstrated yet. The production of extracellular enzymes by Bacilli can be represssible by the addition of glucose in the medium (19). It is likely that some system associated with cell metabolites is involved in the expression of the genes for the extracellular enzymes. It seems possible to consider that the palindromic sequence of amyR2 is a kind of receptor for some factor(s) such as the sacU (pap) and sacQ gene products which may be related to the cell metabolites (20,21,22), because the palindromic sequence is located upstream of the promoter of amyE.

REFERENCES

1. Yamaguchi, K., Matsuzaki, H., and Maruo B. (1969) J. Gen. Appl. Microbiol., 15:97-107.
2. Nomura, S., Yamane, K., Sasaki, T., Yamasaki, M., Tamura, G., and Maruo, B. (1978) J. Bacteriol., 136:818-821.

3. Yamazaki, H., Ohmura, K., Nakayama, A., Takeichi, Y., Otozai, K., Yamasaki, M., Tamura, G., and Yamane, K. (1983) J. Bacteriol., 156: 327-337.
4. Yamane, K., Shiroza, T., Furusato, T., Nakamura, K., Nakazawa, K., Yanagi, K., Yamasaki, M., and Tamura, G. (1985) In Molecular Biology of Microbial Differentiation (J. A. Hoch and P. Setlow, eds) p117-123, Am. Soc. Microbiol., Washington, D.C.
5. Yang, M., Galizzi, A., and Henner, D. (1983) Nucleic Acids Res., 11:237-249.
6. Nicholson, W.L., and Chambliss, G.H. (1985) J. Bacteriol., 161: 875-881.
7. Ohmura, K., Nakamura, K., Yamazaki, H., Shiroza, T., Yamane, K., Jigami, Y., Tanaka, H., Yoda, K., Yamasaki, M., and Tamura, G. (1984) Nucleic Acids Res., 12:5307-5319.
8. Chang, S., and Cohen, S.N. (1979) Molec. Gen. Genet., 168:111-115.
9. O'Callaghan, C.H., Morris, A., Kirby, S.M., and Singler, A.H. (1972) Antimicrob. Agents Chemother., 1:283-288.
10. Shimotsu, H., Kuroda, M.I., Yanofsky, C., and Henner, D.J. (1986) J. Bacteriol., 166:461-471.
11. Wahl, G.M., Stern, M., and Stark, G.R. (1979) Proc. Natl. Acad. Sci. USA. 76:3683-3687.
12. Thomas, P.S. (1980) Proc. Natl. Acad. Sci. USA., 77:5201-5205.
13. Birnboim, H.C., and Doly, J., (1979) Nucleic Acids Res. 7:1513-1523.
14. Southern, E.M. (1975) J. Mol. Biol., 98:503-517.
15. Ohmura, K., Shiroza, T., Nakamura, K., Nakayama, A., Yamane, K., Yoda, K., Yamasaki, M., and Tamura, G. (1984) J. Biochem.(Tokyo), 95:87-93.
16. Shiroza, T., Nakazawa, K., Tashiro, N., Yamane, K., Yanagi, K., Yamasaki, M., Tamura, G., Saito, H., Kawade, Y., and Taniguchi, T. (1985) Gene 34:1-8.
17. Malan, T.P., and McClure, E.R. (1984) Cell 39:173-180.
18. Wu, H.-M., and Crothers, D.M. (1984) Nature (London) 308:509-513.
19. Priest, F.G. (1977) Bacteriol. Rev., 41:711-753.
20. Ayusawa, D., Yoneda, Y., Yamane, K., and Maruo, B. (1975) J. Bacteriol., 124:459-469.
21. Shimotsu, H., and Henner, D.J. (1986) J. Bacteriol., 168:380-388.
22. Amory, A., Kunst, F., Aubert, E., Klier, A., and Rapaport, G. (1987) J. Bacteriol. 169:324-333.