(S7-D)

THE REGULATION OF ALPHA-AMYLASE SYNTHESIS IN THE STRAINS BEARING degU MUTATION.

Misun Won and Glenn. H. Chambliss
Department of Bacteriology, University of Wisconsin-Madison, Wisconsin 53706

Introduction

In B. subtilis, the regulatory mutations including degU (1, 18, 20, 22, 25), degQ (11), degR (13) and hpr (9) result in pleiotropic phenotypes. Strains bearing a degU(Hy) mutation not only hyperproduce extracellular drgradative enzymes such as α-amylase, alkaline and neutral protease and levansucrase but also show phenotypic changes such as the disappearance of a membrane fraction component, loss of competence, formation of filamentous cells and a decrease in lytic activity Mutations at the degU locus can increase the steady-state level of sacB (levansucrase structural gene) mRNA, probably by increasing the rate of transcription initiation (22). Deletion studies of upstream of the aprE (alkaline protease=subtilisin) promoter implicated the possible existance of an upstream transcriptional activation site at which expression of aprE is mediated by the degU(Hy)32, degQ(Hy)36 and hpr-97 gene products in response to a variety of nutritional conditions (6).

To study regulation of amylase synthesis in degU(Hy) mutants, plasmid p5' B10 (Fig.1) in which amyR1 directs the promoterless cat-86 gene was introduced into strains bearing degU32(Hy) and degU100(Hy) mutations.

1. α-Amylase production.

Amylase specific activities in culture supernatants were assayed in 168 and degU(Hy) mutants at t_2. As seen in Table 1., QB13 (degU32(Hy)) had less amount of -amylase activity, QB 136 (degU32(Hy)) produced 1.5 fold more, and QB157 (degU100(Hy)) produced 2 fold more than wild type 168 in the absence of glucose. α-Amylase

synthesis was repressed by the addition of glucose in all strains. Intracellular α-amylase activities measured after lysing cells were about 1/10 of extracellular α-amylase secreted into media in all strains. Intracellular α-amylase activities in *degU*(Hy) mutants were not much higher than 168 and the activities were not quite repressed by glucose in any of the strains suggesting the existence of basal pool of α-amylse probably involved in maturation for transient period of time before secretion or in other regulation.

The levels of α-Amylase proteins detected by western blotting using IgY against α-amylase and rabbit IgG against IgY-alkaline phosphatase conjugate corresponded to the specific activities measured by KI-I_2 method in both intracellular and extracellular enzymes of 168 and *deg*(Hy) mutants (data not shown). This result supports that no larger pool of accumulated enzymatically inactive α-amylase protein exists inside cells of *degU*(Hy) strains.

2. ***amyE*** **mRNA levels.**

The *amyE* mRNA levels in *degU*(Hy) strains were compared to that of 168 by S1 mapping protection analysis (Fig.1). The *amyE* mRNA levels in all strains were repressed by glucose. The mRNA levels of *degU*(Hy) strains were at least 4 times, 6-7 times in QB157(*degU*100(Hy)) higher than they were in 168. It was suprising because amylase activity was correlated well with the amount of amylase protein detected by western blotting.

Chloramphenicol acetyltransferase (CAT), an intracellular enzyme, was chosen to study the effect of *degU* on CAT synthesis. Introduction of plasmid p5' B10 carrying an *amyE-cat-86* transcriptional fusion into *degU*(Hy) strains had no effect on α-amylase activity and *amyE* mRNA pool size (Fig.3). Two *amyE* mRNA bands, about 2.2 and 1.55 knt, were detected by northern blot. 2.2 knt corresponded to full length of

amyE mRNA. It was assumed that 1.55 knt was degradation product of amyE or new transcript initiated from the promoter-like region located 640 bsae pairs downstream determined by nucleotide sequence analysis.

3. CAT (chloramphenicol acetyltransferase) production.

The plasmid-born CAT activities were measured in 168(p5' B10), QB136(p5' B10) and QB157(p5' B10) (Table 2). QB136(p5' B10) and QB157(p5' B10) had 3 and 7 times respectively as much activity as 168(p5' B10) in the absence of glucose and in each case glucose repressed acivites. The increased CAT activity is close to that of amyE mRNA were detected in deg(Hy) strains. This result seems to be reasonable since the synthesis of intracellular CAT enzyme is controlled by amyR1. The cat-86 mRNA levels of 168(p5' B10) and QB157 (p5' B10) were measured by northern blot experiment (Fig.3). Because cat-86 mRNA have processing at the 3'- and 5'-end (1), 3 major bands (810, 720 and 620 nt) were detected when BclI-HindIII fragment was used as a probe. QB 157(p5' B10) produces 7 folds more cat-86 mRNA which corresponded well with the CAT activity of the strains.

Dicussion

In the cloned degU, there are two open reading frames, degS and degU (7, 12). Both strain 1A95 (degU32(Hy)) and 1A165 (degU32(Hy)) are mutated in degU and the 1A206(degU100(Hy)), in degS. The N-terminus sequence of degU gene product exhibits homology with signal transduction family via phospo-transfer such as ntrB-ntrC, cheA-cheB, spoOA-spoOF system(8, 16). The C-terminus of the degU gene product had homology to other positive regulatory proteins (4, 10). These homologies suggest that phosphorylated degU may regulatelate transcription from certain genes of degradative enzymes. Some silmilarities in amino acids sequence and concerved domain of degS to other modulator

proteins by computer analysis hypothesize that degS gene product may be a modulator protein of degU gene product (7, 12) The degU(Hy) acts by stimulating transcription in sacB in levansucrease (6, 22), aprE promoter of subtilisin (6) even if it is not clear whether or not degU gene product interact directly (6). When compared the corresponding A-T rich region sequences, amyR had less homology with aprB or sacB (25). The observation degU100(Hy) which has mutation in degS had more dramatic effect on amyE mRNA than degU32(Hy) in degU locus supports that degS gene product may modulate the function of degU gene product as suggested previously (7).

Early studies reported that strains bearing degU(Hy) mutation produced 2-3 fold more α-amylase (23) but 6-10 times more alkaline protease or levansucrase (3, 25) than wild type 168. Why degU(Hy) mutation has less effect on α-amylase synthesis than on alkaline protease or levansucrease as measured by enzyme activity (25) is an open question. The limited secretion hypothesis would dictate that a pool of α-amylase should accumulate in degU(Hy) mutants. Estimation of the pool size based on amylase protein content by western blotting revealed essentially no difference between wild type and degU(Hy) mutants. The amyE transcription could be stimulated to an extent similar to that of sacB or aprE but the amyE mRNA not be translated efficiently. This disparity between mRNA level and enzyme production implies that post-transcriptional step is limiting α-amylase accumulation in the degU(Hy) mutants.

It is possible that gene expression can be controlled at the level of mRNA degradation, that is, chemical decay and translational or functional inactivation. It is not clear whether modulation of RNA stability leads to a change in protein synthesis or and whether the intracellular concentration of mRNA does reflect the amount of protein made. For certain specific

genes there may be autoregulation of translation by the gene product (17). Also, translational or functional inactivation of mRNA by processing at the 5'-end has been reported (2, 21). The REP (repetitive extragenic palindromic) sequence which can stabilize upstream RNA in independently of any other sequences, by protection from 3'-5' exonuclease attack. The REP stabilized mRNA was translationally active leading to increase in relative gene expression in the maltose operon (14, 15). It was shown that Salmonella typhimurium cells containing hisJ mRNA plus the 3'stem-loop (REP) sequence produce at least 10 times more hisJ mRNA than strain carring hisJ without REP sequence express only 2 times more hisJ protein than the REP minus strain (14, 25). The reduced funtional half-life of hisJ mRNA in REP-deleted hisJ strain led to conclusion that only a small portion of the REP-stabilized hisJ mRNA was functionally active (14). In contrast, in the constructed cat-galK operon, the REP sequence caused three fold increase in upstream cat mRNA , which leads to equivalent increase in CAT activity (14). Additionally, when the ermC leader sequence which is involved in mRNA stabilization in the presence of Erythromycin in Staphylococcus aureus was inserted upstream of E. coli lacZ and B. pumulis cat-86, both hybrid transcripts were chemically stabilized in the presence of Em (19). CAT activity increased 8 fold after induction with Em, however, no increase in β-galactosidase activity was dectected suggesting that erm-lacZ transcript was not functionally stabilized.

The modulation of mRNA affecting gene expression seems to be different in different mRNA species (14, 15, 19). The amyE mRNA may be a similar case to hisJ or lacZ transcript. This suggests that only a small portion of the increased amyE mRNA is translated in the degU(Hy) strains possibly due to translational functional inactivation. In contrast, cat-86 mRNA derived

from the amyR1-cat-86 transcriptional fusion was directly translated without inactivation resulting in a more direct correspondence between the level of CAT mRNA and CAT activity detected. Our observation of the lack of correspondence between the level of amyE mRNA and amylase activity may be specific to amylase since alkaline protease and levansucrase activity increase proportionately with their mRNAs in degU(Hy) strains.

The mechanism by which the mRNA becomes functionally inactive is not known. It could become modified so that it is no longer accessible to the translational machinery. Other possible modifications include binding of cellular proteins, folding of the transcript or specific modification of nucleotides.

References

1. Aymerich, S., G. Gonzy-Trebou, and M. Steinmetz 1986. J. Bacteriol. 166:993-998.
2. Cannistraro, V.J., and D. Kennell. 1985. J. Mol. Biol. 182:241-248.
3. Chambert, R., and M.-F. Petit-Glatron. 1984. J. Gen. Microbiol. 130:3143-3152.
4. Cole, S. T., F. Start, W. G. Beattie, and R. E. Moses. Gene 42:201-208.
5. Dod, B., and G. Balassa. 1978. Mol. Gen. Genet. 163:57-63.
6. Henner, D. J., E. Ferrari, M. Perego, and J. A. Hoch. 1988. J. Bacteriol. 170:296-300.
7. Henner, D. J., M. Yang, and E. Ferrari. 1988. J. Bacteriol. 170:5102-5109
8. Hess, T. F., N. Oosawa, N. Kaplan, and M. I. Simon. 1988. Cell 53:79-87.
9. Higerd, T. B., J. A. Hoch, and J. Spizizen. 1972. J. Bacteriol. 112:1026-1028.
10. Holland, S. K., S. Cutting, and J. Mandelstam. 1987. J. Gen. Microbiol. 133:2381-2391.
11. Kunst, F., M. Pascal, J. Lepesant-Kejzlarova, J-A. Lepesant, A. Billauts, and R. Dedonder.

1974. Biochimie 56:1481-1489.
12. Kunst, F., M. Debarbouille, T. Msadek, M. Young, C. Mauel, D. Karamata, A. Klier, G. Rapoport, and Dedonder. 1988. J. Bacteriol 170:5093-5101.
13. Nagami, Y., and T. Tanaka. 1986. J. Bacteriol. 166:20-28.
14. Newbury, S. F., N. H. Smith, E. C. Robinson, T. D. Hiles, and C. F. Higgins. 1987. Cell 48:297-310.
15. Newbury, S. F., N. H. Smith, and C. F. Higgins. 1987. Cell 51:1131-1143.
16. Ninfa, A. J., and B. Magasanik. 1986. Proc. Natl. Acad. Sci. U.S.A. 83:5909-5913.
17. Nomura, M., R. Gourse, and G. Baughman. 1984. Biochem. 53:75-117.
18. Ruppen, M. E., G. L. van Alstine, and L. Band. 1988. J. Bacteriol. 170:136-140.
19. Sandler, P.. 1989. Ph.D. Thesis. University of Wisconsin.
20. Seiguchi, J., N. Tanaka, and O. Okada. 1975. J. Bacteriol. 168:380-388.
21. Shen, V., M. Cynamon, B. Daugherty, H.-F.. Kung, and D. Schlessinger. 1981. J. Biol. Chem. 256:1896-1902
22. Shimotsu, H., D. J. Henner. 1986. J. Bacteriol. 168:380-388.
23. Steinmetz, M., F. Kunst, and R. Dedonder. 1976. Mol. Gen. Genet. 148:281-285.
24. Stern, M. J., G. F-L. Ames, N. H. Smith, E. C. Robison, C. F. Robinson and C. F. Higgins. 1984. Cell 37:1015-1026.
25. Yoneda, Y., and B. Maruo. 1975. J. Bacteriol. 142:48-54.

Table 1. α-amylase specific activity (U) and relative amount of amyE mRNA of 168 and degU (Hy) mutants.

Strain	Amylase S.A. (U.)[a]					
	Extracellular		Intracellular		amyE mRNA (%)[b]	
	-Glc	+Glc	-Glc	+Glc	-Glc	+Glc
168(W.T.)	118	20	15	7	100	2
QB13(sacU(h)32)	78	15	6	6	430	2
QB136(sacU(h)32)	173	21	9	8	N.D.	N.D.
QB157(sacU(h)100)	238	33	22	8	780	3

[a] Each strain was grown in Difco in the absence or presence of glucose added at 60 klett units. The culture supernatant at t_2 were assayed for extracellular amylase activity. Intracellular amylase activity was determined by assaying lysed cells. The average of 3 growth was obtained as amylase specific activity

[b] Each strain was grown in Difco in the absence or presence of glucose to t_1 at which amyE mRNA reaches the maximum level. The amyE mRNA level was determined by S1 nuclease protection experiment and each transcript was scanned by laser densitometer and standardized to be compared.

Table 2. CAT specific activity measured at t_2.

Strain	Mutation	CAT S.A. (mU)	
		-Glc	+Glc
168(p5'αB10)	W. T.	65	6
QB13(p5'αB10)	sacU(h)32	119	25
QB136(p5'αB10)	sacU(h)32	176	17
QB157(p5'αB10)	sacU(h)100	490	93

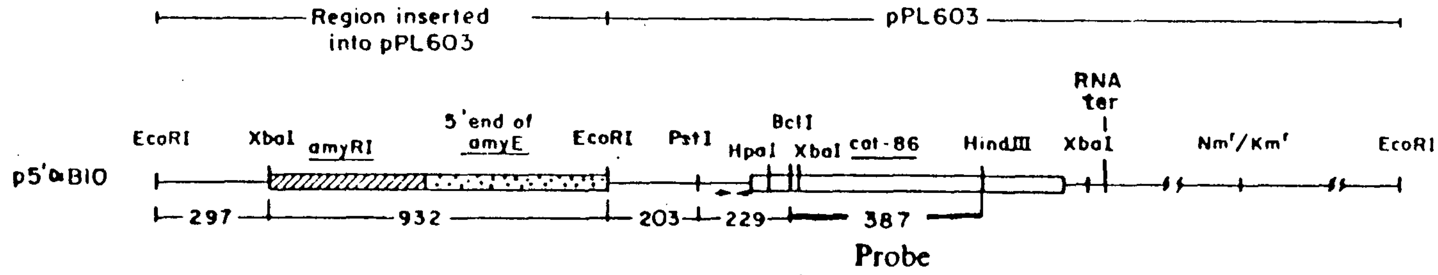

Fig. 1. Transcriptional fusion of amyR and cat-86. BclI-HindIII fragment was used as a probe to detect cat-86 mRNA in northern blot analysis.

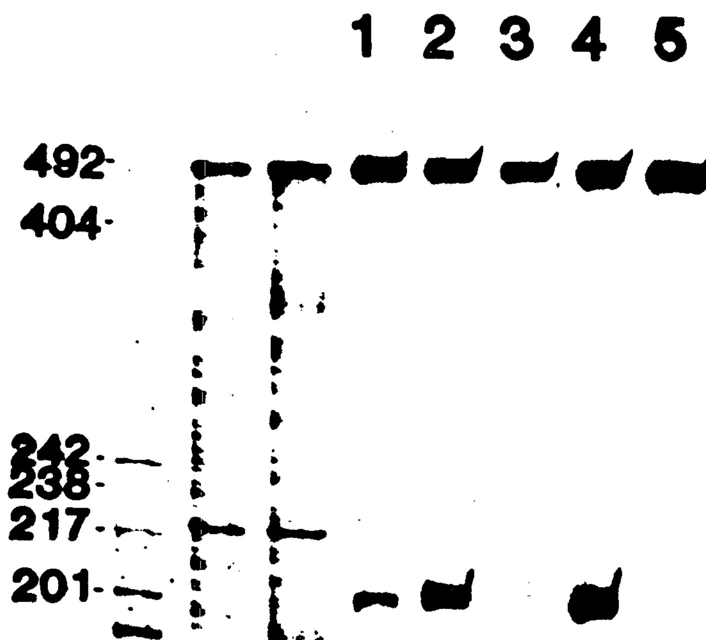

Fig. 2. Determination of relative amount of *amyE* mRNA in 168 and *degU*(Hy) mutants. Total cellular RNAs isolated from cells at t_1 in the presence (lanes 3 and 5) and in the absence (lanes 1, 2 and 4) was used to determine *amyE* mRNA by S1 nuclease protection experiment. Lane 1, 168 ; lanes 2 and 3, QB 13, lanes 4 and 5, QB157.

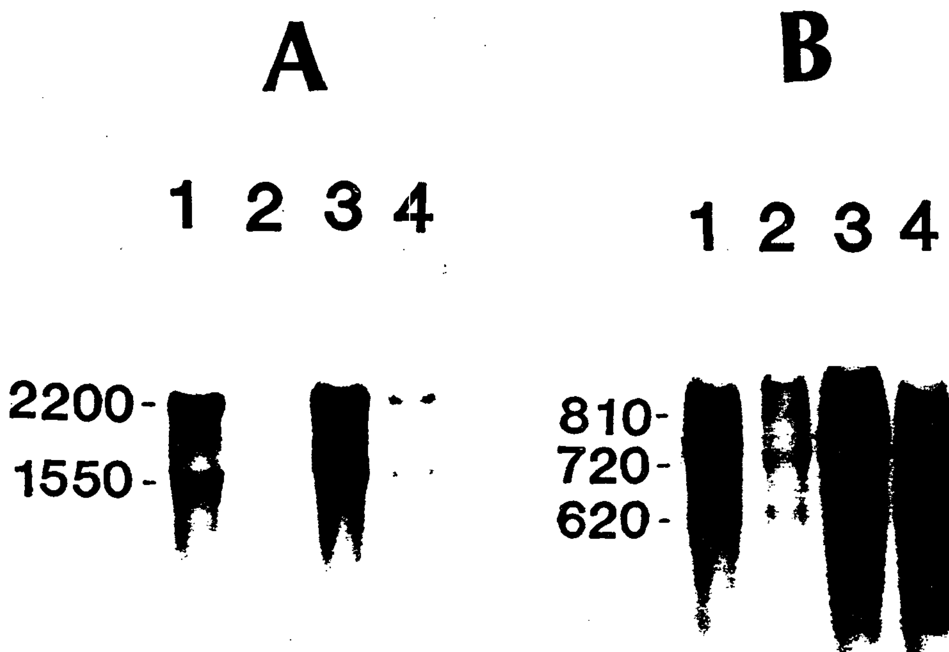

Fig. 3. Relative levels of amyE mRNA and cat-86 mRNA in 168(p5' B10) and QB157 (p5' B10) at t_1. Total cellular RNAs were isolated and used for northern blot experiment to detect amyE mRNA and cat-86 mRNA. The 417 bp of SalI-EcoRI DNA fragment and 387 bp of BclI-HindIII were labelled and used as probes for amyE mRNA and cat-86 mRNA respectively.
A. Relative amount of amyE mRNA.
Lanes 1 and 3, no glucose added ;
lanes 2 and 4, glucose added :
lanes 1 and 2, 168(p5' B10) ;
lanes 3 and 4, QB157(p5' B10).
B. Relative amount of cat-86 mRNA.
Lanes 1 and 3, no glucose added ;
lanes 2 and 4, glucose added :
lanes 1 and 2, 168(p5' B10) ;
lanes 3 and 4, QB157(p5' B10).